STUDIES ON THE BIOSYNTHESIS OF EXTRACELLULAR POLYSACCHARIDES IN THE ENTEROBACTERIACEAE

by

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SUMMARY

The exopolysaccharide produced by many strains of *Escherichia coli* K12, and various *Salmonella* spp., has been identified as Colanic acid, on the basis of the identification of the monosaccharide components (glucose, galactose, fucose and glucuronic acid), the quantitative analysis of these components, and the sensitivity of the exopolysaccharide to several bacteriophage-induced depolymerase enzymes which have been prepared. It has been shown that a considerable number of *Salmonella* spp., previously considered non-mucoid, can be induced to form exopolysaccharide when grown in the presence of p-fluorophenylalanine (PFA), and the exopolysaccharide has been identified as Colanic acid. In direct analogy with the situation which has been reported by other authors to exist with strains of *Escherichia coli* K12, this has led to the suggestion that most, if not all, of the *Salmonellae* have the genetic ability to synthesise Colanic acid, but that the synthesis is frequently genetically repressed. Several other members of the *Enterobacteriaceae*, including *Shigella* and *Aerobacter* spp., have been examined for the ability to produce Colanic acid, and the occurrence of Colanic acid within the *Enterobacteriaceae* discussed.

The nucleotide pools of certain mucoid strains of *Escherichia coli* K12, *Salmonella typhimurium* and *Aerobacter cloacae*, have been examined, and in all cases the nucleotide

sugars, uridine diphosphate glucose (UDPG), uridine diphosphate glucuronic acid (UDPGA), uridine diphosphate galactose (UDPGal), and guanosine diphosphate fucose (GDPF) were detected, which has led to the suggestion that these nucleotide sugars are involved in the synthesis of Colanic acid. The involvement of three of these nucleotide sugars has been further indicated by the examination of three mutant strains of Escherichia coli K12, one UDPG pyrophosphorylase-less, one UDPG-4-Epimerase-less, and one UDPG dehydrogenase-less, all of which proved to be non-mucoid, even in the presence of PFA.

The levels of the four nucleotide sugars believed to be involved in Colanic acid synthesis, and of the enzymes involved in their synthesis have been examined in a number of mucoid strains, and compared with the levels found in certain repressed strains which had been shown to be mucoid only in the presence of PFA. It has been found that the levels of UDPG, UDPGal, and the enzymes involved in their synthesis, are substantially the same in both types, but that the levels of UDPGA, GDPF, and the enzymes UDPG dehydrogenase, guanosine diphosphate mannose (GDPM) pyrophosphorylase, and GDPF synthetase, were very much higher in mucoid strains, indicating that the control of Colanic acid synthesis is at the nucleotide sugar level. On growing repressed strains in the presence of PFA, the levels of UDPGA and GDPF detected, approached those to be found in mucoid strains. The possible existence of an operon under the control of a regulator gene, containing genes coding for certain key

enzymes involved in Colanic acid synthesis, such as UDPG dehydrogenase, GDPM pyrophosphorylase, and GDPF synthetase, has been suggested.

Attempts to isolate possible lipid-linked intermediates in Colanic acid synthesis from whole cells have not been successful, and attempts to achieve the cell-free synthesis of Colanic acid, using various enzyme preparations, and the four nucleotide sugars believed to be involved, have also met with lack of success.

C O N T E N T S

INTRODUCTION

P A R T I

GENERAL INTRODUCTION

The overwhelming majority of all naturally occurring carbohydrates exist in the form of compounds of high molecular weight, and a very large number of polymeric carbohydrates are encountered, whether from plant, animal, or microbiological source. Historically, the type simplest to investigate was the relatively uncomplicated homopolysaccharide species, where the molecule is built up by polymerisation of a single sugar unit. Thus the most abundant of the naturally occurring homopolysaccharides, such as starch, glycogen, and cellulose, were examined, mainly from a structural viewpoint, but also with regard to the biosynthetic reactions involved in their formation. Recently, many new techniques have been developed, both chemical and biological, and the new world of complex heteropolysaccharides has been entered with great success. Some of the heteropolysaccharides investigated, and under investigation, may have many different components, and often, not only has the structure with all its complexities been elucidated in considerable detail, also the mode of biosynthesis of the polymer has been authenticated. Today, much interest centres round even more complex mixed polymers, where carbohydrates are found combined in some type of linkage with protein, peptides, or

lipids. That such an advance in our knowledge and ambition should have taken place in a relatively short time, stems directly from the great improvement in the techniques first developed in the early days of polysaccharide research, and the instigation of a sophisticated array of new, mainly biological techniques, which have made the examination of the vastly more complex world of heteropolymers less formidable a prospect.

Many milestones in the field exist, but if one were to single out one or two of the most significant, then the recognition of the central role played by nucleotide sugars in carbohydrate metabolism, due to the pioneer work of Leloir and his group in the 1950s, probably was responsible for the upsurge in interest and understanding of polysaccharide biosynthesis, which led to another major discovery during the last few years by several laboratories, of lipid-linked oligosaccharides, which serve as intermediates in the biosynthesis of certain heteropolysaccharides.

Discoveries such as these have reorientated and revitalised concepts of polysaccharide synthesis, and may lead to a reappraisal of much of the early work in the field.

Initial studies on the biosynthesis of polysaccharides were severely hampered by a complete ignorance of the precursors involved. A considerable amount of information was available about degradative mechanisms, and particularly a great deal was known about hydrolytic enzymes which would cleave polysaccharides or oligosaccharides to their monomers. It was thought that,

given the correct conditions, these enzymes would work in reverse, so kinetic studies on such carbohydrases were carried out with a view to ascertaining the correct conditions for reversal of the enzymic reactions, but in order to obtain appreciable synthesis of carbohydrate, very large quantities of monosaccharides had to be present, far more than known to occur *in vivo*.

This period of essentially catabolic investigation was brought to a close by an important investigation carried out by Cori and his group (Cori and Cori, 1936; Cori, 1940), who demonstrated that an enzyme, entirely different from the hydrolytic type, was responsible for the degradation of glycogen in liver to produce ∝-D-glucose-1-phosphate (G-1-Ⓟ). Soon afterwards it was found (Cori and Cori, 1940) that this enzyme, phosphorylase, present in liver, muscle, and yeast, will reverse the reaction, forming glycogen-like material from ∝-D-glucose-1-P, the reaction however requiring large amounts of G-1-Ⓟ and any accumulation of P_i pushing the reaction in the reverse direction,

$$n\ \text{G-1-Ⓟ} \rightleftharpoons \text{1-4 glucose}_n + P_i$$

A similar enzyme was demonstrated in higher plants, catalysing a reaction producing an amylose-like polymer (Hanes, 1940; 1941). These reactions constitute some of the first examples of the biosynthesis of a polymer taking place *in vitro*, and as such are historically very significant. The biosynthesis of the microbial polysaccharide, dextran, was also under

investigation at this time (Hehre, 1941; Hehre and Sugg, 1942) and a cell-free system was obtained from Leuconostoc mesenteroides, catalysing the formation of dextran from a disaccharide precursor, sucrose, in a reaction which was irreversible,

$$n \text{ sucrose} \longrightarrow 1\text{-}6 \text{ glucose}_n + n \text{ fructose}$$

A similar system was to be described in Aerobacter levanicum, for the synthesis of levan (Hestrin, Avineri-Shapiro and Ascher, 1943). A phosphorylase-type system in bacteria was under investigation (Doudoroff, Barker and Hassid, 1947), which catalysed the biosynthesis of the disaccharide, sucrose, from α D-glucose-1-Ⓟ and D-fructose, the enzyme being specific towards α D-glucose-1-Ⓟ, but much less specific towards D-fructose, which could be replaced by a number of other sugars, and non-glycosidic compounds, showing the reaction to be essentially a transfer mechanism, where the glucose moiety of G-1-Ⓟ is transferred to an acceptor molecule. In the light of this discovery, and the possibility that the polysaccharide systems investigated involved a similar mechanism, a new concept of polysaccharide biosynthesis evolved (Doudoroff, Barker and Hassid, 1947), the concept of transglycosidation, where the carbohydrate part of a glycosyl donor is transferred to an acceptor, forming a new glycoside, the then known donors being sugar phosphates or disaccharides. This intuitive concept was to prove well founded in later years, after a period of stasis for a few years until the discoveries of the

FIGURE 1 UDPG

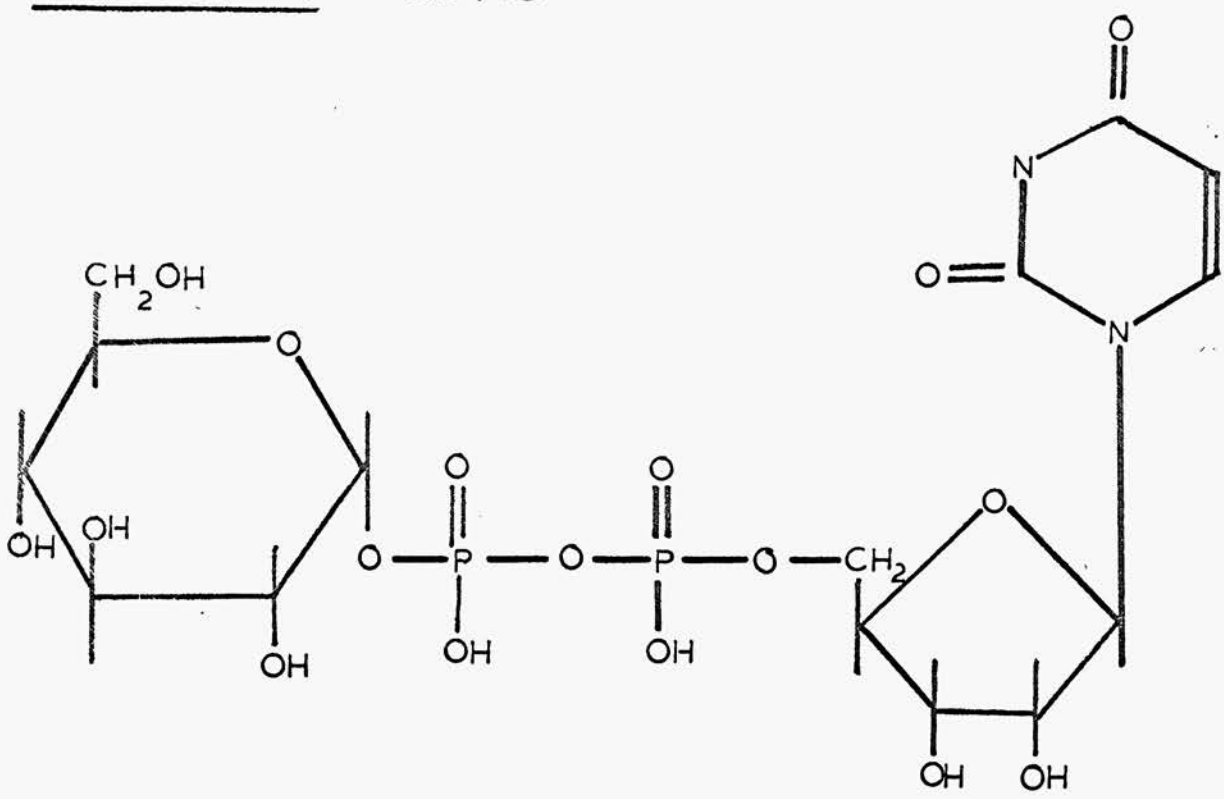
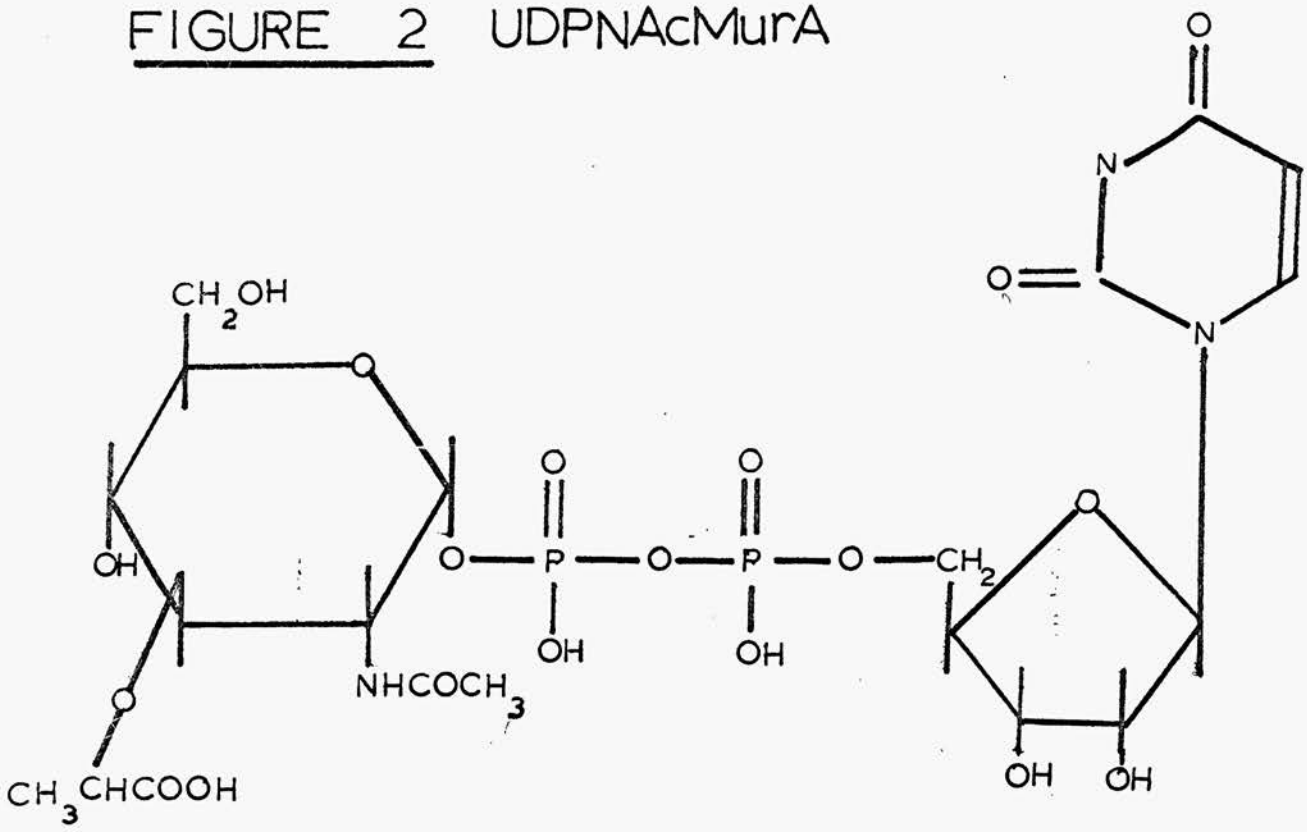


FIGURE 2 UDPNAcMurA



early 1950s. In the interim, further studies on the biosynthesis of commonly occurring homopolysaccharides were notable for their lack of success, for it soon became apparent that these systems already examined, involving disaccharides or phosphate esters, were a few specialised cases, and not to be taken as model systems sensu lato. The basic ignorance of the precursors involved in polysaccharide biosynthesis was not to be resolved until the important role of a new class of compound was realised, both in the building up of complex polysaccharides, and as an agent in the interconversion of monosaccharides.

Leloir and his co-workers were investigating the metabolism of lactose in Saccharomyces fragilis (Caputto et al., 1948; Leloir et al., 1948), and discovered that cell-free extracts of the organism catalysed the phosphorylation of the galactose moiety to give α-D-galactose-1-phosphate (gal-1-Ⓟ) which was converted to α-D-glucose-1-Ⓟ, this conversion requiring a heat-stable co-factor (Cardini et al., 1949; Trucco et al., 1948). Eventually this co-factor was isolated and its structure elucidated, proving it to be a derivative of α-D-glucose phosphate, where the hemiacetal carbon atom is joined by an ether linkage to the terminal phosphoric acid group of uridine diphosphate, the substance being given the name, uridine diphosphate glucose (UDPG) (Cardini et al., 1950) (Figure 1). At about the same time, Park and Johnston (Park and Johnston, 1949) started to work on some compounds that accumulated in penicillin treated cells of Staphylococcus

aureus. One of the compounds was subsequently identified (Park, 1952a; 1952b; 1952c) as a uridine diphosphate derivative of the new sugar, muramic acid (Figure 2) with a side chain of several amino acids attached to some point in the sugar molecule. These new sugar derivatives were collectively given the name "sugar nucleotides", the name embracing a class of compound containing a sugar or sugar derivative, esterified by a glycosidic hydroxyl group to the terminal phosphate residue of a nucleoside 5′ diphosphate (quoted in Neufeld and Hassid, 1963).

Leloir further went on to elucidate the role played by UDPG in the interconversion of G-1-Ⓟ and Gal-1-Ⓟ, and found that the conversion involved two steps (Leloir, 1951).

$$\text{UDPG} + \text{gal-1-Ⓟ} \rightleftharpoons \text{UDPGal} + \text{G-1-Ⓟ} \qquad \text{A}$$

$$\text{UDPGal} \rightleftharpoons \text{UDPG} \qquad \text{B}$$

Reaction B revealed for the first time the role of a sugar nucleotide in a sugar transformation, one of the many reactions they were later found to participate in.

The possibility that UDPG might have some role other than in the interconversion of glucose and galactose, was suggested by Buchanan et al. (1952) on purely hypothetical grounds. Park (1952c; 1952d) had previously suggested that the compounds accumulated by S. aureus might act as amino acid or peptide donors in protein synthesis, and that penicillin blocked the system in some way. Buchanan et al. (1952) drew a parallel between UDPG and Acetyl CoA which acted in the transfer and incorporation of acyl groups, being of the view that compounds

FIGURE 3 SYNTHESIS OF O-AMINO PHENOL-β-D-GLUCOSIDURONIC ACID

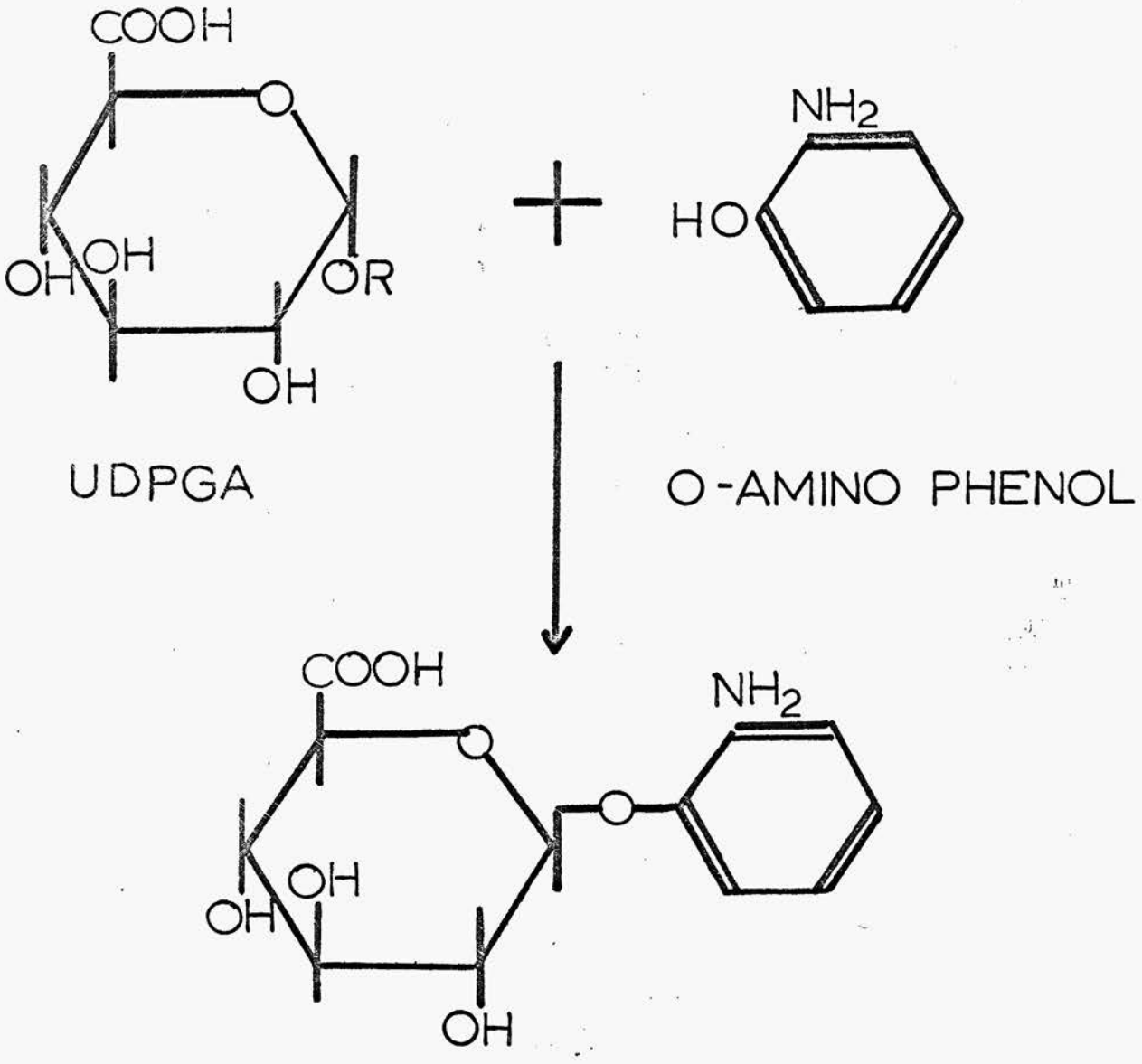
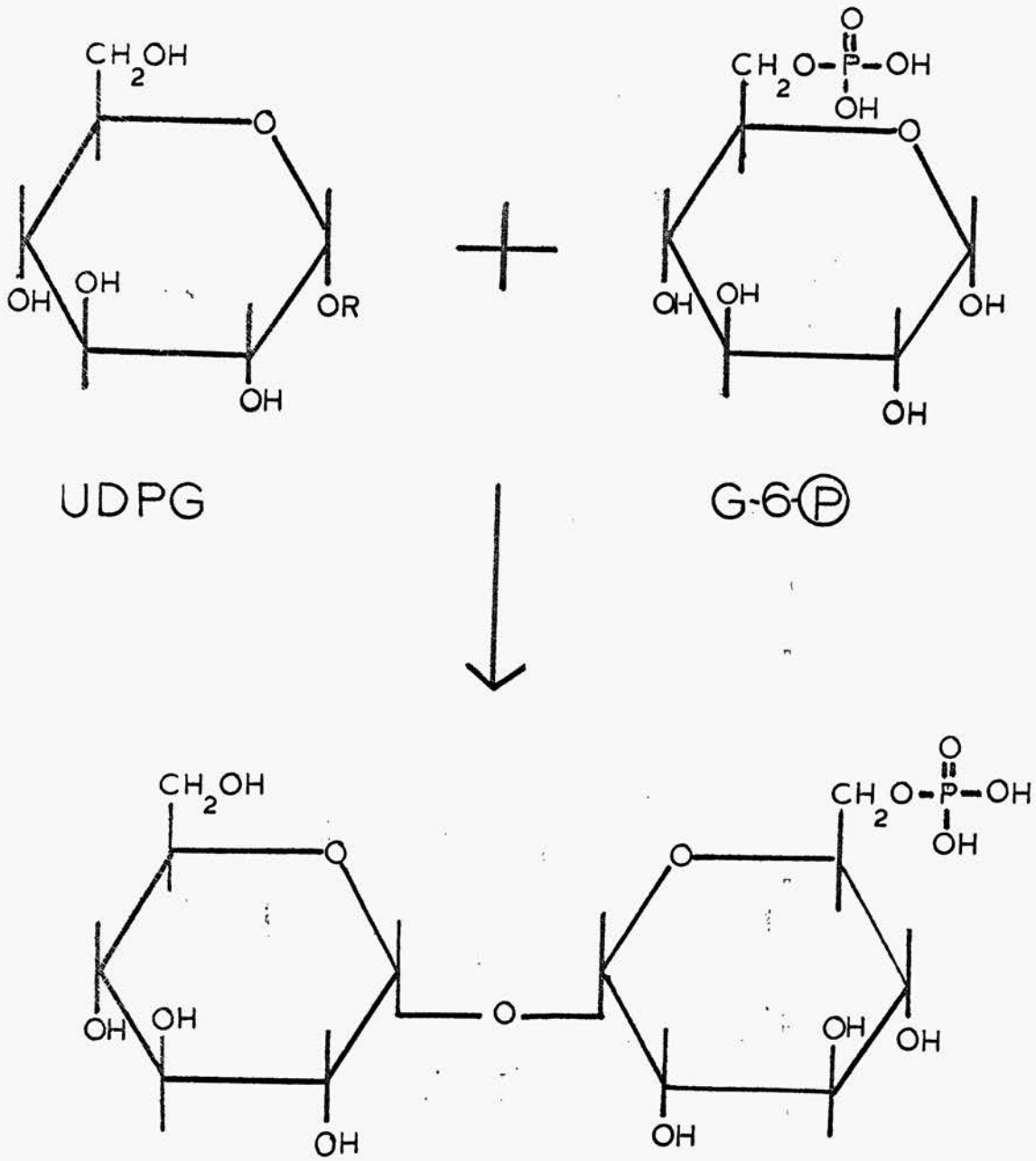


FIGURE 4 SYNTHESIS OF TREHALOSE-Ⓟ



of the UDPG type might not only be concerned with the transformation of sugars, but also with their subsequent incorporation into polysaccharides. However, it was left to Dutton and Storey (1953) to provide the conclusive evidence that such compounds could act as glycosyl donors. These workers (Dutton and Storey, 1951) had reported that the synthesis of O-aminophenol β-D-glucosiduronic acid in liver required the presence of boiled liver extract. Subsequently, they (Dutton and Storey, 1953) identified the active principle as a sugar nucleotide compound, uridine diphosphate glucuronic acid (UDPGA), and they were able to demonstrate that in liver homogenates, the glycosyl moiety of UDPGA was transferred to O-aminophenol or menthol to form a β-glucosiduronide (Figure 3), the first in vitro evidence for the participation of nucleotide sugars in glycoside synthesis. Shortly afterwards, the involvement of UDPG in the synthesis of a disaccharide phosphate, trehalose phosphate (Leloir and Cabib, 1953), was reported (Figure 4), and nucleotide sugar participation in the biosynthesis of a polysaccharide, hyaluronic acid (Glaser and Brown, 1955). The sugar nucleotides of Park, which had been suggested as intermediate in protein synthesis, took on a new meaning following reports on the composition of the Gram-positive bacterial cell wall (Salton, 1952a; 1952b). On the basis of similarities between these sugar nucleotides, and certain components of the cell wall, it was suggested (Park and Strominger, 1957) that these compounds might be intermediates

in cell wall biosynthesis, accumulated because of a blockage in the system caused by penicillin. In later years this was shown to be so (Chatterjee and Park, 1964). On the basis of this early work, it seemed likely that the biosynthesis of saccharides in general, involved glycosyl transfer from sugar nucleotides, a remarkable vindication of the old theory of transglycosylation from phosphate esters, even if fortuitous.

Such a theory was shown to be founded on strong thermodynamic grounds with the calculation of the energy available from the hydrolysis of the glucose-phosphate bond of UDPG at -7600 calories (Leloir, Cardini and Cabib, 1960), considerably greater than that of any other phosphate ester donor in saccharide synthesis, any equilibrium in a biosynthetic system clearly favouring the formation of complex saccharides. The energy available from hydrolysis of the phosphate bond of α-D-glucose-1-Ⓟ is -4800 calories, suggesting that the enzyme, phosphorylase, is principally catabolic in function (Oesper, 1961) supported by the discovery that in certain diseases (Hers, 1959; Schmid, Robbins and Trout, 1959) the glycogen content is elevated above the normal level, whereas the amount of phosphorylase present is either markedly diminished or altogether absent, indicating that another pathway must exist for the formation of glycogen.

The dextran and levan synthesising systems in bacteria and fungi involve only a net redistribution of glycosidic links, not an increase, and may be an efficient method for poly-

TABLE 1: SUGAR NUCLEOTIDES

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
D-glucose	UDP	yeast	(1)	UDPG pyrophosphorylase	yeast	(11)
		other fungi	(2)(3)		bacteria	(12)(13)
		bacteria	(4)(5)		plants	(14)(15)
		plants	(6)(7)		animals	(16)(17)
		animals	(8)(9)(10)			
	GDP	fungi	(18)	GDPG pyrophosphorylase	animals	(19)
		animals	(19)		plants	
	ADP	plants	(20(21)	ADPG pyrophosphorylase	plants	(22)
					bacteria	(13)(23)(24)
	dADP	-	[illegible]	dADPG pyrophosphorylase	plants	(23)
	dTDP	bacteria	(25)	dTDPG pyrophosphorylase	bacteria	(13)(26)(27)
	CDP	-		CDPG pyrophosphorylase	bacteria	(13)(27)
	IDP	-		IDPG pyrophosphorylase	plants	(23)
D-galactose	UDP	yeast	(28)	UDPGal pyrophosphorylase	yeast	(33)
		other fungi	(3)		plants	(34)
		bacteria	(29)		animals	(35)
		plants	(6)(7)	UDPG-4-Epimerase	yeast	(36)(37)
		animals	(30)(31)(32)		plants	(34)
					animals	(38)
					bacteria	(39)(36)
				galactose-1-P uridyl transferase	yeast	(36)
					bacteria	(12)
					plants	(40)
					animals	(41)

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
D-galactose	GDP	animals	(42)	-		
	ADP	plants	(43)	-		
	dTDP	bacteria	(25)	dTDPGal pyrophosphorylase	bacteria plants	(44) (45)
				dTDPG-4-Epimerase	bacteria	(46)(47)
	dUDP	-		dUDPGal pyrophosphorylase	plants	(45)
L-galactose	GDP	plants animals	(7) (46)	-		
D-mannose	GDP	yeast other fungi plants animals	(47) (48) (7) (5)(49)(50)	GDPM pyrophosphorylase	yeast bacteria animals	(51) (52) (49)
	dTDP	fungi	(53)	dTDPG-2-Epimerase	fungi	(53)
	ADP	plants	(43)	-		
D-glucuronic acid	UDP	bacteria	(4)(5)	UDPG dehydrogenase	plants bacteria animals	(57) (56) (58)
		plants	(7)			
		animals	(54)(55)	UDPGA pyrophosphorylase	plants	(59)
				UDPGalA-4-Epimerase	bacteria plants	(56) (60)

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
D-galacturonic acid	UDP	bacteria plants	(5) (61)	UDPGA-4-Epimerase	bacteria plants	(56) (60)
				UDPGalA pyrophosphorylase	plants	(59)
D-mannuronic acid	GDP	plants	(62)	GDPM dehydrogenase	bacteria	(63)
L-iduronic acid	UDP	-		UDPGA-5-Epimerase	animals	(64)
L-guluronic acid	GDP	plants	(62)	-		
6,deoxy-D-glucose	dTDP	bacteria	(65)			
6,deoxy-D-mannose (D-rhamnose)	GDP	bacteria	(66)	-		
6,deoxy-D-talose (D-talomethylose)	GDP	bacteria	(66)	-		
6,deoxy-L-galactose (L-fucose)	GDP	bacteria animals	(67) (68)	oxidoreduction of GDPM	bacteria	(142)
	dTDP	bacteria	(65)			

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
6,deoxy-L-mannose (L-rhamnose)	dTDP	bacteria	(69)(70)(71)	oxidoreduction of dTDPG	bacteria	(72)(65)
	UDP	plants bacteria	(73) (74)			
4,keto-6,deoxy-D-glucose	dTDP	bacteria	(65)(75)	oxidoreduction of dTDPG	bacteria	(76)(65)(77)(78)
	CDP	-		oxidoreduction of CDPG	bacteria	(79)(80)(81)
4,keto-6,deoxy-D-mannose	GDP	-		oxidoreduction of GDPM	bacteria	(82)
3,6,dideoxy-L-galactose (colitose)	GDP	bacteria	(83)	oxidoreduction of GDPM	bacteria	(84)(82)
3,6,dideoxy-D-galactose (abequose)	CDP	bacteria	(85)	oxidoreduction of CDPG	bacteria	(75)
3,6,dideoxy-L-mannose (ascarylose)	CDP	-		oxidoreduction of CDPG	bacteria	(75)(81)
3,6,dideoxy-D-glucose (paratose)	CDP	bacteria	(86)	CDP tyvelose-2-Epimerase	bacteria	(87)(88)
				Oxidoreduction of CDPG	bacteria	(75)

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
3,6,dideoxy-D-mannose (tyvelose)	CDP	bacteria		CDP paratose-2-Epimerase	bacteria	(87)(88)
D-glucosamine	UDP	–		$UDPGNH_2$ pyrophosphorylase	animals	(89)(13)
				$UDPGalNH_2$-4-Epimerase	animals	(91)
	ADP	–		$ADPGNH_2$ pyrophosphorylase	bacteria	(13)
	dTDP	–		$dTDPGNH_2$ pyrophosphorylase	bacteria	(13)(90)
	CDP	–		$CDPGNH_2$ pyrophosphorylase	bacteria	(13)
D-galactosamine	UDP	–		$UDPGNH_2$-4-Epimerase	animals	(91)
N-acetyl-D-glucosamine	UDP	bacteria	(4)(5)	$UDPNAcGNH_2$ pyrophosphorylase	yeast	(96)
		yeast	(92)		bacteria	(13)(97)
		other fungi	(2)		animals	(97)(17)
		plants	(93)			
		animals	(94)(95)(9)(10)	$UDPNAcGalNH_2$-4-Epimerase	bacteria	(98)
	ADP	–		$ADPNAcGNH_2$ pyrophosphorylase	bacteria	(13)

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
N-acetyl-D-glucosamine	dTDP	-		dTDPNAcGNH$_2$ pyrophosphorylase	bacteria	(13)
				dTDPNAcGalNH$_2$-4-Epimerase	animals plants	(96) (91)
	UDP	bacteria plants animals	(98) (99) (94)(97)(100)	UDPNAcGNH$_2$-4-Epimerase	bacteria	(98)
	dTDP	-		dTDPNAcGNH$_2$-4-Epimerase	animals plants	(96) (91)
4,amino-4,6-dideoxy-D-glucose (viosamine)	dTDP	-		oxidoreduction and amination of dTDPG	bacteria	(101)
4,acetylamino-4,6-dideoxy-D-glucose	dTDP	bacteria	(102)	oxidoreduction, amination and acetylation of dTDPG	bacteria	(76)
4,acetylamino-4,6-dideoxy-D-galactose	dTDP	bacteria	(25)(65) (103)	oxidoreduction, amination and acetylation of dTDPG	bacteria	(76)
3,acetylamino-3,6-dideoxy-D-galactose	dTDP	bacteria	(46)	oxidoreduction, amination and acetylation of dTDPG	bacteria	(46)

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
lactose ↑	GDP	animals (milk)	(128)	-		
$NAcGNH_2$ ↑ galactose	UDP	animals (milk)	(129)	-		
NANA						
$NAcGNH_2$ ↑ L-fucose	UDP	animals (milk)	(130)	-		
$NAcGalNH_2$-4-SO_4	UDP	animals (hen oviduct)	(131)(132)	-		
$NAcGalNH_2$--6-Ⓟ-galactose	UDP	animals (hen oviduct)	(131)(132)			
dihydroxyacetone	UDP	bacteria	(133)	-		
choline	CDP	animals yeast	(135)(137) (138)	phosphorylcholine pyrophosphorylase	animals	(139)
	dCDP	animals	(134)			

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
2,acetylamino-4, amino-2,4, 6-trideoxyhexose	UDP	bacteria	(104)	oxidoreduction, amination and acetylation of $UDPNAcGNH_2$	bacteria	(104)
N-acetyl muramic acid peptides	UDP	bacteria	(105)(106)	Condensation of phosphenolpyruvate with $UDPNAcGNH_2$, followed by stepwise addition of amino acids	bacteria	(107)(108)
N-acetyl neuraminic acid	CMP	bacteria	(109)	CMPNANA pyrophosphorylase	bacteria animals	(110) (111)
N-glycol neuraminic acid	CMP	-		CMPN-glycol NA pyrophosphorylase	animals	(111)
2,keto-3, deoxyoctonic acid	CMP	-		CMPKDO pyrophosphorylase	bacteria	(112)
2,3,diphospho glyceric acid	AMP	animals	(113)	-		
D-ribose	dTDP	fungi	(114)	-		
L-arabinose	UDP	plants	(115)	UDP arab pyrophosphorylase	plants	(116)

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
L-arabinose				UDPX-4-Epimerase	plants	(116)(117)
D-xylose	UDP	plants fungi animals		UDPGA decarboxylase	plants fungi animals bacteria	(60) (118) (119) (118)
				UDP arab-4-Epimerase	plants	(117)
ribitol	CDP	bacteria	(120)(122)	CDP ribitol pyrophosphorylase	yeast bacteria plants	(121) (121) (121)
glycerol	CDP	bacteria	(120)	CDP glycerol pyrophosphorylase	yeast bacteria plants	(121) (121) (121)
glycerol-P-glycerol	CDP	bacteria	(123)	-		
D-fructose	GDP	fungi	(124)	-		
	UDP	plants	(125)	-		
D-glycero-D-manno-heptose	GDP	yeast	(126)	GDP D-glycero-D-manno-heptose pyrophosphorylase	yeast	(126)
6,deoxy-3C methyl-2.0 methyl-L-aldoheptose (vinelose)	CDP	bacteria	(127)	-		

Sugar moiety	Nucleotide moiety	Isolation source	Reference	Synthesis observed	Enzyme source	Reference
ethanolamine	dCDP	animals	(136)			
	CDP	animals	(137)	phosphorylethanolamine pyrophosphorylase	animals	(140)
diglycerides	CDP	-		phosphatic acid pyrophosphorylase	animals	(141)

saccharide synthesis in the case of organisms which encounter preformed saccharides in their natural environment. The systems described are certainly biosynthetic systems, and are therefore not likely to be catabolic in any way, and as such must occupy a special niche in any discussion of polysaccharide biosynthesis, if for no other reason than they helped to formulate the overall theory of transglycosidation.

At our present level of knowledge it would be fair to say that if a certain sugar is found in a polysaccharide, then it can also be found as a nucleotide sugar, and as a consequence of this, studies on the biosynthesis of a particular polysaccharide often begin with the investigator trying to isolate, and identify, nucleotide sugar precursors of the biosynthetic system. Indeed, sometimes the discovery of a sugar nucleotide has preceded that of the corresponding polymer, as in the case of the isolation and identification of cytidinediphosphate glycerol and cytidinediphosphate ribitol (Baddiley et al., 1956; Baddiley, Buchanan and Carss, 1957) which suggested the existence of glycerol and ribitol polymers and led to their discovery (Baddiley, 1962). To date, more than eighty different sugar nucleotides have been isolated from plants, animals, and bacteria, or as a result of enzymic reactions derived from these sources (Table I). Sugar derivatives of all the common pyrimidines and purines are known, and the list also includes compounds which do not strictly adhere to the definition quoted, having non-carbohydrate residues attached to

nucleotides, suggesting that in general terms, the nucleotide moiety could be considered as a convenient highly energised carrier for some unit, whether it be a sugar, sugar derivative, or other compound, indicating an applicability of the method outwith the realms of carbohydrate biochemistry.

Most naturally occurring monosaccharides are usually found as constituents of complex compounds, or bound in the form of nucleotide sugars, and are seldom found as free sugars, which are generally only produced as a result of degradative processes. When sugars are supplied preformed to organisms, in most cases they enter the general metabolism by conversion, and are not directly incorporated into sugar nucleotide compounds (Ginsburg, 1964). Thus, animals cannot use D-glucuronic acid directly for the synthesis of glucuronides (Douglas and King, 1953) or L-fucose for the synthesis of mucopolysaccharides (Draper and Kent, 1963), and plants are unable to use D-xylose for the synthesis of xylans (Hassid, Neufeld and Feingold, 1959). However, conversely, some higher plants have the necessary enzymes for the direct incorporation of several sugars such as D-glucuronic acid, D-galactose, and L-arabinose into sugar nucleotides (Hassid, Neufeld and Feingold, 1959), and in the case of the bacteria there is the example of the direct incorporation of galactose into uridine diphosphate galactose via the so far unique transferase system described by Leloir (1951), a necessary step in the metabolism of this sugar.

In most instances, nucleotide sugars are synthesised by an

enzyme of the pyrophosphorylase type. In 1953 it was shown (Munch-Peterson et al., 1953) that UDPG could be formed by yeast extracts from α-D-glucose-1-phosphate and uridine triphosphate. The reaction is considered to involve a transfer of a uridyl group from pyrophosphate to a sugar-1-phosphate, with release of pyrophosphate (PP),

$$UTP + G\text{-}1\text{-}\textcircled{P} \rightleftharpoons UDPG + PP$$

The reaction is freely reversible, but in vivo, hydrolysis of the pyrophosphate bond may drive the reaction to the right. Derivatives of guanosine, thymidine, adenosine and cytidine, are formed in an analagous manner from various sugar phosphates and the corresponding nucleoside triphosphate (Table I). The sialic acids, N-acetyl neuraminic acid (NANA), and 2 keto, 3 deoxy octonic acid (KDO) are found to be formed as nucleotide derivatives by a somewhat different type of pyrophosphorylase. In these cases, the transfer of the nucleotidyl group from the nucleoside triphosphate, takes place with a free sugar, rather than a sugar phosphate,

$$KDO + CTP \rightleftharpoons CMPKDO + PP$$ (Ghalambor and Heath, 1963)

$$NANA + CTP \rightleftharpoons CMPNANA + PP$$ (Roseman, 1962)

So far, these are the only examples of nucleotide monophosphate sugars encountered, although another nucleoside monophosphate compound, adenosine monophate 2,3 diphosphoglyceric acid, has been described in some types of animal blood (Hashimoto et al., 1961) and as such, they too, do not fit the original definition of nucleotide sugar.

Once formed, the glycosyl moiety of sugar nucleotides is capable of undergoing many transformations. The first of these to be encountered was the interconversion of UDPG and UDPGal, involving an epimerisation of carbon atom 4 (Leloir, 1951). The reaction requires nicotinamide adenine dinucleotide (NAD) as a co-factor, so it is believed that the epimerisation is brought about by an oxidation and reduction at C4 (Turner and Turner, 1958), but efforts to isolate the postulated 4 keto sugar nucleotide intermediate were unsuccessful, and it is suggested that the intermediate exists only transiently, bound to the enzyme surface. Analogous C4 epimerisations occur in reactions derived from many different sources (Table 1),

dTDPG $\rightleftharpoons$ dTDPGal (Tinelli, Michelson and Strominger, 1963)

$UDPNAcGNH_2 \rightleftharpoons UDPNAcGalNH_2$ (Glaser, 1959)

UDPGA $\rightleftharpoons$ UDPGalA (Feingold, Neufeld and Hassid, 1960; Smith et al., 1959)

$dTDPNAcNH_2 \rightleftharpoons dTDPNAcGalNH_2$ (Maley and Maley, 1959)

UDParabinose $\rightleftharpoons$ UDPxylose (Ankel et al., 1967)

Although C4 epimerisations are most commonly encountered, other epimerisations exist. The only known direct interconversion of a D-mannose and a D-glucose residue occurs via a C2 epimerisation in Streptomyces griseus (Baddiley and Blumson, 1960). One other C2 epimerisation is known, that of the interconversion of CDP 3-6-dideoxy-D-glucose (CDP paratose) with CDP 3-6-dideoxy-D-mannose (CDP tyvelose) in the Salmonellae (Matsuhashi and

Strominger, 1965). In skin, where the major mucopolysaccharide is chondroitin sulphate B, which has as its uronic acid component iduronic acid, a C5 epimerisation takes place,

UDPGA ⇌ UDPIA (Jacobson and Davidson, 1962).

Presumably GDP-L-guluronic acid isolated from seaweed (Lin and Hassid, 1966) is derived from GDP mannuronic acid in a similar C5 epimerisation, although this has not yet been shown.

Oxidation of sugar nucleotides at C6 gives rise to uronic acid derivatives, and the formation of UDPGA from UDPG is a widespread reaction (Table I). GDP mannuronic acid is formed from GDPM in an analagous reaction so far restricted to the bacteria (Preiss, 1962). The mechanism of oxidation has been investigated (Simonart, Selo and Kirkwood, 1967) in the UDPGA system. The oxidation is unusual in that it involves a two step process, brought about by a single enzyme, an unidentified intermediate being formed initially in the NAD dependent reaction. The intermediate appeared to have an altered nucleotide moiety, rather than a semi-oxidised sugar component, and was quickly further oxidised to UDPGA.

UDPGA is known to be decarboxylated to give UDPXylose, in an enzyme reaction widely distributed in tissues, the only reaction of this type so far discovered (Feingold, Neufeld and Hassid, 1960; Ankel and Feingold, 1966; Ankel et al., 1967). This reaction is interesting, since it seems to provide the only method of synthesising activated pentoses, apart from the direct incorporation of L-arabinose into a sugar nucleotide

FIGURE 5 TRANSFORMATIONS OF dTDPG

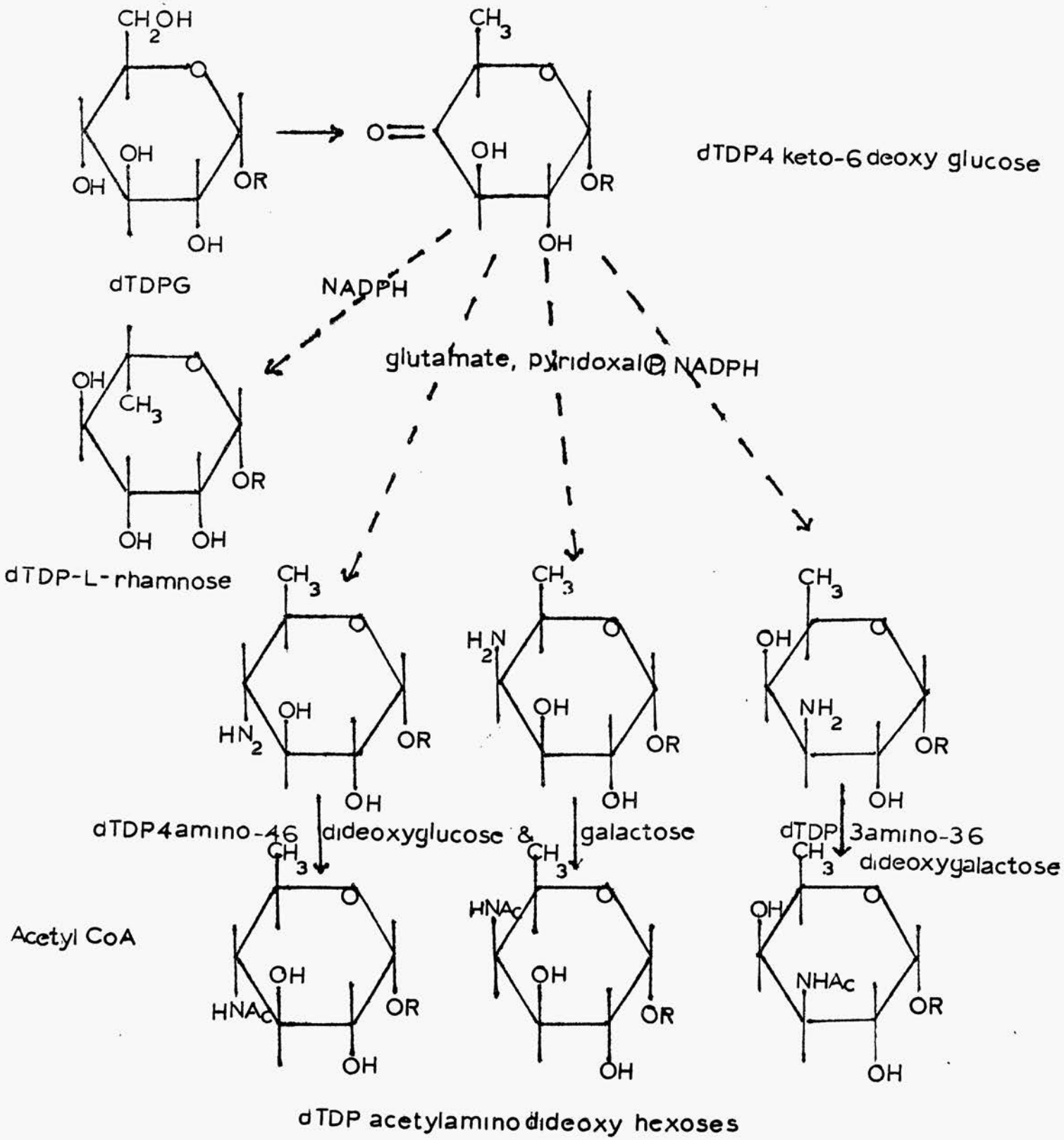
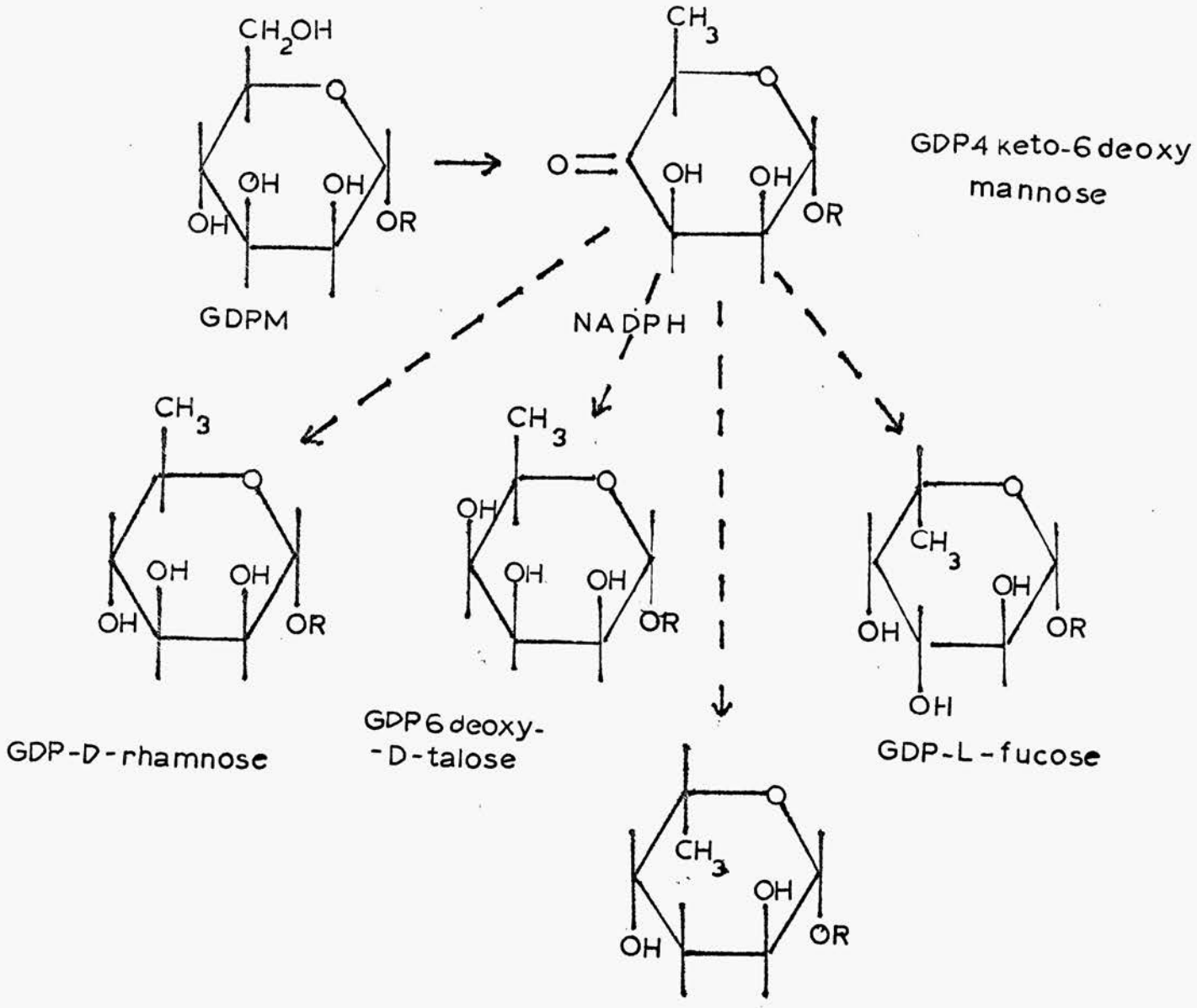


FIGURE 6 TRANSFORMATIONS OF GDPM



in higher plants (Hassid, Neufeld and Feingold, 1959).

More complex conversions of nucleotide sugars take place in the formation of deoxysugars. The first step involves dehydration, followed by NAD dependent oxidation to yield a nucleotide 4 keto-6 deoxysugar, which can then undergo further rearrangement, including epimerisation at C3, C4,or C5, amination, acetylation, and a final stereospecific reduction by NADPH to yield any of a number of deoxy, dideoxy or trideoxysugar nucleotides, perhaps aminated and acetylated. The key compounds in the formation of deoxysugars are dTDPG, CDPG, and GDPM, the former giving rise to several compounds via dTDP-4 keto-6 deoxy-D-glucose (Figure 5), including dTDP-L-rhamnose (Glaser and Kornfeld, 1961; Okazaki et al., 1962), dTDP-4 N-acetyl amino-4,6 dideoxyhexoses and dTDP-4 amino-4,6 dideoxyhexoses (Matsuhashi and Strominger, 1964), and probably to dTDP-3 N-acetyl amino-3,6 dideoxyhexoses (Volk and Ashwell, 1963) and in an analagous series of reactions GDPM gives rise to GDP-L-fucose (Ginsburg, 1960; 1961), GDP-colitose (GDP-3,6 dideoxy-L-galactose) (Heath and Elbein, 1962), GDP-6 deoxy-D-mannose (GDP-D-rhamnose) and GDP-6 deoxy-D-talose (Markowitz, 1964), via GDP-4 keto-6 deoxy-D-mannose (Figure 6). CDPG gives rise to CDP-3,6 dideoxy-D-galactose (CDP abequose) (Matsuhashi, Matsuhashi and Strominger, 1966b), CDP-3,6 dideoxy-D-glucose (CDP paratose) (Matsuhashi, Matsuhashi and Strominger, 1966b; Matsuhashi and Strominger, 1965), CDP-3,6 dideoxy-L-mannose (CDP ascarylose) (Matsuhashi, Matsuhashi and Strominger, 1966b;

FIGURE 7 TRANSFORMATIONS OF CDPG

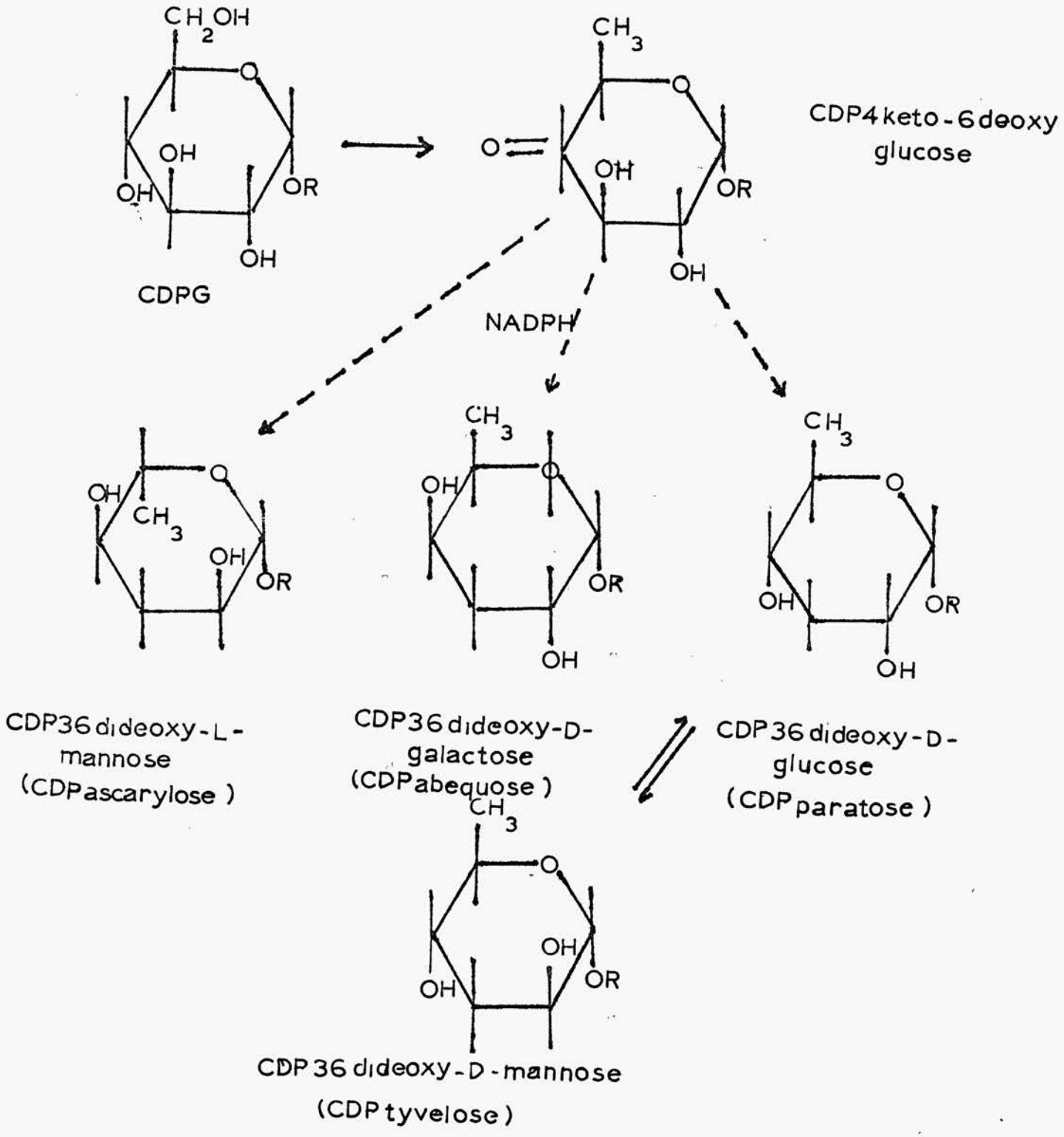
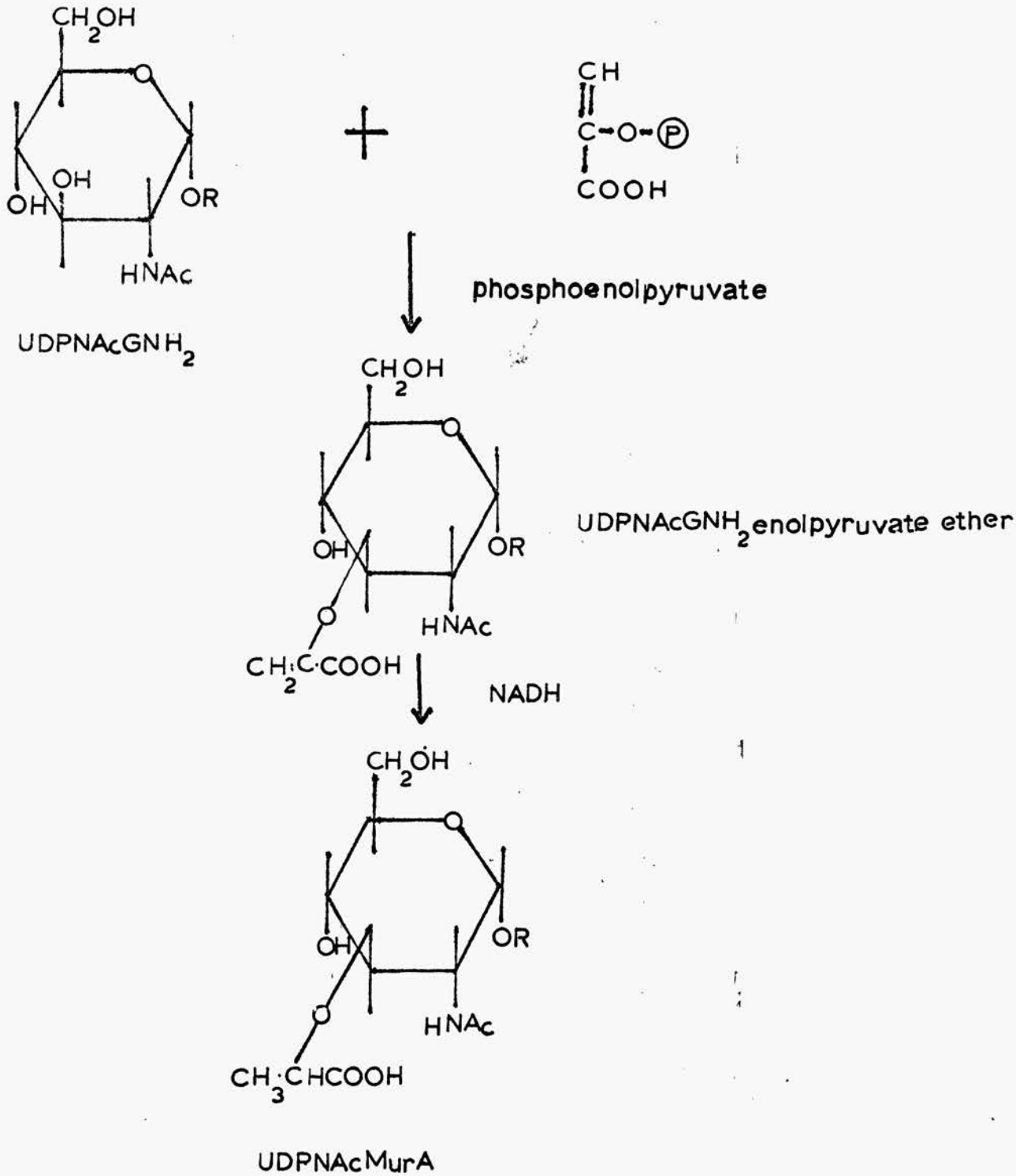


FIGURE 8 SYNTHESIS OF UDPNAcMurA



Matsuhashi et al., 1964) and CDP-3,6 dideoxy-D-mannose (CDP tyvelose) (Matsuhashi, Matsuhashi and Strominger, 1966a) (Figure 7). The enzymic reactions are not known in detail in the cases cited, particularly as to how the molecular rearrangement takes place, but it has been suggested that a series of alternate enolisations and ketonisations take place after the original dehydration, allowing epimerisation of carbon atoms to occur (Okazaki et al., 1962; Matsuhashi, Matsuhashi and Strominger, 1966b; Ginsburg, 1961).

Another important precursor in sugar nucleotide interconversions is UDPNAcGNH$_2$, which is involved in the synthesis of the extraordinary diaminosugar nucleotide described by Distler and his co-workers (Distler, Kaufman and Roseman, 1966) participating in a similar series of molecular rearrangements as those involved in dideoxysugar formation. The sugar nucleotide peptides of Park (1952a; 1952b; 1952c) are formed from UDPNAcGNH$_2$ by S. aureus extracts, which catalyse condensation of phosphoenolpyruvate with the N-acetyl group of the molecule (Strominger, 1958) followed by stepwise addition of amino acids to the carboxyl group of the lactyl ether so formed, in the order, L alanine, D glutamate, L lysine (Ito and Strominger, 1962a), and a final addition of D alanyl D alanine (Ito and Strominger, 1962b) (Figure 8). Those bacteria whose cell wall contains meso-diaminopimelic acid (DAP) instead of L-lysine, incorporate DAP in its place (Strominger, Ito and Threnn, 1961).

Liberation of sugars from sugar nucleotides seldom occurs,

and there are only a few reported instances. One such case is the uridyl transferase reaction described in the metabolism of galactose (Leloir, 1951) which is a necessary step in intermediary metabolism, but this presumably involves no loss in energy. Liberation of a sugar from a sugar nucleotide occurs in the formation of N-acetyl mannosamine ($NAcMNH_2$) in animals (Ghosh and Roseman, 1965). In this strange reaction, so far not described in bacteria, an epimerisation at C2 occurs in $UDPNAcGNH_2$, accompanied by liberation of the sugar moiety from the molecule,

$$UDPNAcGNH_2 \rightleftharpoons NAcMNH_2 + UDP.$$

Other reactions are known which result in liberation of a sugar or a sugar phosphate, and corresponding loss of energy, but so far they are of limited occurrence, and the precise function of such reactions is not clear. An enzyme found in yeast, sugar nucleotide phosphorylase (Cabib,, Carminetti and Woyskovsky, 1965) is active with a large number of sugar nucleotides in the following reaction:

$$XDP\ sugar + \textcircled{P} \rightleftharpoons XDP + sugar\text{-}1\text{-}\textcircled{P}.$$

A specific ADPG phosphorylase catalysing a similar reaction is found in wheat germ (Dankert, Concalves and Recondo, 1964) and in bacteria, a pyrophosphatase catalysing the breakdown of CDP glycerol has been described (Glaser, 1965). One other specific enzyme is known, also found in yeast, GDPG glucohydrolase, which catalyses the breakdown of GDPG in an as yet unique reaction,

$$GDPG \rightleftharpoons GDP + G.$$

All of these reactions may somehow be involved in the control of sugar nucleotide production, and it may be that many other tissues will show similar mechanisms contributing towards a polysaccharide biosynthesis control mechanism, operating at the sugar nucleotide level.

The concentration of sugar nucleotides in different organisms varies over a wide range, from 370 uM UDPG/kg. wet weight in yeast (Caputto et al., 1950) to very low values such as 0.1 uM UDPGalA/kg. mung bean (Neufeld and Feingold, 1961), and the concentration of sugar nucleotide observed may have no relationship to its metabolic significance. The in vitro synthesis of nucleotide sugars may also have little significance, unless accompanied by isolation of the particular nucleotide sugar from the same source, since the possibility must be considered that the synthesising enzyme may not be entirely specific, and, in vitro, may synthesise several sugar nucleotides when presented with the apposite precursors. Cell-free extracts of Azotobacter will catalyse the formation of eleven different sugar nucleotides (Kimata and Suzuki, 1966) when presented with the necessary sugar phosphates and nucleoside triphosphates, including four different nucleotide derivatives of glucose, three of N-acetylglucosamine, and four of glucosamine, surely an unnecessarily wide range for the organism to produce in vivo. Similarly, investigation into the biosynthesis of nucleotide sugars in Arthrobacter (Shen and Preiss, 1965) revealed seven different nucleotide derivatives

of glucose, and on the basis of changes in activity during purification of the enzymes, it was suggested that only UDPG, dTDPG and ADPG were synthesised by separate enzymes, and that the other four nucleotide derivatives were produced as a result of non-specific synthesis by these three enzymes. Conversely, isolation of a particular nucleotide may have little significance under certain circumstances, since from a derivative of E. coli, a mixture of nucleotide sugars was found, including dTDPF and dTDP-6 deoxyglucose (Okazaki et al., 1962). No enzymic method for the synthesis of these sugars was found in extracts of the organism, and it is suggested that they were formed by non-specific reduction of another sugar nucleotide found, dTDP-4 keto-6 deoxyglucose, either during the isolation or purification procedure.

Of the sugar nucleotides listed in Table I, clearly there are some compounds which occur in practically all material investigated. Notably UDPG, GDPM, and UDPNAcGNH$_2$ are universally found, along with the necessary enzymes for their synthesis, indicating that they are central intermediates in the synthesis of many saccharides. They are also important in view of their precursor relationships with other monosaccharides, since UDPG can give rise to UDPGal, UDPGA, UDPIA, UDPGalA, UDPX, UDPA, and GDPM to GDPMA, GDPF, GDPColitose, GDP-D-rhamnose, GDP-D-talomethylose, and presumably to GDP-L-galactose and GDP-L-guluronic acid (Table I). It is interesting to note that only the two C2 epimerisations are known, and that by

epimerisation at C3, C4, or C5, UDPG could give rise to many hexoses of D configuration at C2, and GDPM to many with L configuration at C2, so that a biosynthetic separation exists between the two forms of sugar (Ginsburg, 1964), the only direct interconversion between D-glucose and D-mannose reported being the C2 epimerase of S. griseus (Baddiley et al., 1962). $UDPNAcGNH_2$ may not be so significant as a precursor, but it is involved in the synthesis of $NAcMNH_2$ (Ghosh and Roseman, 1965), $UDPNAcGalNH_2$, the N-acetylmuramic peptides involved in cell-wall biosynthesis, and the diamino sugar synthesised by Pneumococcus (Table I).

Despite the widespread occurrence of certain nucleotide sugars, there appear to be a large number of monosaccharides which can be attached to any one of several nucleotides, and in some instances reactions occur with a monosaccharide attached to a particular nucleoside diphosphate in one organism, and an analagous reaction takes place with the same monosaccharide component attached to a different nucleotide in another. This is illustrated by the C4 epimerisation of $NAcGNH_2$ on a UDP unit in many organisms (Glaser, 1959; Maley and Maley, 1959), and also on dTDP in some cases (Maley and Maley, 1959; Glaser and Brown, 1955). The dTDPG-4-epimerase of plants and bacteria (Neufeld, 1962) has the same function as the ubiquitous UDPG-4-epimerase. It may be that an organism has a choice of nucleotide carrier, perhaps as a method of separating closely related metabolic pathways which may involve the same monosaccharide

component at some stage (Ginsburg, 1964) since if the same nucleotide derivative were a precursor in several pathways, a complex control mechanism would be required. This biochemical separation is evident in the case of the formation of the more complex deoxysugars. α-D-glucose, attached to a uridine nucleotide, undergoes the many transformations already discussed, but other nucleotide derivatives of glucose are widely found in the bacteria. In the biosynthesis of L-rhamnose (Glaser and Kornfeld, 1961) and the acetylamino dideoxyhexoses (Matsuhashi and Strominger, 1964; Volk and Ashwell, 1963), the precursor molecule is dTDPG, whereas in the biosynthesis of abequose (Matsuhashi, Matsuhashi and Strominger, 1966b), paratose (Matsuhashi and Strominger, 1965), tyvelose (Matsuhashi, Matsuhashi and Strominger, 1966a) and ascarylose (Matsuhashi et al., 1964), the precursor is CDPG. Since all the pathways involve conversion of a glucose residue to a 4 keto-6 deoxyglucose residue, clearly it would be an advantage for an organism, such as one of the Salmonellae, which may have a cell wall containing glucose, galactose, rhamnose, and one of the 3-6 dideoxyhexoses (Luderitz, Jann and Wheat, 1968), to be able to control the synthesis of these independently, and presumably this would be easier to do if the biosynthetic pathways were easily separable.

The bacteria provide an interesting and plentiful supply of new sugar nucleotides, and the discovery of a new compound leads to much speculation as to its function. New and interesting amino sugars in particular, have been found in the

last few years as nucleotide derivatives, such as 4 N-acetyl amino-4,6 dideoxyhexoses (Okazaki et al., 1962; Matsuhashi and Strominger, 1967), 3 N-acetyl amino-3,6 dideoxyhexose (Volk and Ashwell, 1963), and 2 N-acetyl amino-4 amino-2,4,6 trideoxyhexose (Distler, Kaufman and Roseman, 1966). 4 N-acetyl amino-4,6 dideoxyhexoses have been described in polysaccharides isolated from strains of Escherichia coli (Jann and Jann, 1967), and Chromobacterium violaceum (Wheat, Rollins and Leatherwood, 1962). 4 amino-4,6 dideoxyhexoses (Stevens, Blumbergs and Daniker, 1963; Okazaki, Okazaki and Kuriki, 1960), 2 amino-2,6 dideoxyhexose (Crumpton and Davies, 1958), and 4 N-acetyl amino-2 amino-2,4,6 trideoxyhexose (Sharon and Jeanloz, 1960) have also been isolated from bacterial sources, but in general terms, the isolation of deoxyamino sugars from polysaccharides has seldom been reported. Isolation of such compounds as nucleotide derivatives has led to a reappraisal of the techniques used for the examination of polysaccharides, since often the conditions employed to examine a polysaccharide cause destruction of the amino sugar. Indeed Stevens et al. (1964a) have reported that five strains of Pasteurella, seven strains of Salmonellae, and two of Escherichia are capable of synthesising nucleotide derivatives of 4 N-acetyl amino-4,6 dideoxyhexoses. This suggests that the existence of such amino sugars may be widespread among the bacteria, despite the very few reported instances of their isolation from polysaccharides. One can only assume that using the apposite

Nucleotide donor	Synthesis observed	Enzyme source	Reference
UDPG	+ quercetin → quercetin glucoside	plants	(62)
	+ phenyl β-D-glucoside → phenyl β-D-gentobioside	plants	(63)
dUDPG	+ quercetin → quercetin glucoside	plants	(62)
dTDPG	+ quercetin → quercetin glucoside	plants	(62)
UDPRhamnose	+ quercetin glucoside → rutin	plants	(62)
dTDPRh	+ quercetin glucoside → rutin	plants	(62)
UDPGA	+ O-aminophenol → O-aminophenol-β-D-glucosiduronic acid	animals	(64)(65)
	and other acceptors such as phenols, alcohols, carboxylic acids, aromatic amines	animals	(66)(67)(68)(69)(70)
	+ quercetin → quercetin glucosiduronic acid	plants	(71)

Nucleotide donor	Synthesis observed	Enzyme source	Reference
UDPGal	+ other isomers of sphingosine → galactosylated	animals	(55)
GDPM	+ diglyceride ⟷ mannosyl diglyceride ↓ mannosyl-mannosyl diglyceride	bacteria	(56)
dTDPRh	+ 3 hydroxydecanoyl CoA → rhamnolipids	bacteria	(57)
CDP choline	+ diglyceride → lecithin	animals	(97)
	+ Nacyl D threo transsphingosine → sphingomyelin	animals	(98)
CDP ethanolamine	+ diglyceride → phosphatidyl ethanolamine	animals	(99)
CDP diglycerides	+ inositol → phosphatidyl inositol	animals	(100)
	+ glycerophosphate → phosphatidyl glycerophosphate	animals bacteria	(100)(101)
	+ L serine → phosphatidyl serine	bacteria	(101)
	Aromatics, etc.		
UDPG	+ hydroquinone → hydroquinone-β-D-glucoside	plants animals	(58) (59)
	+ O-aminophenol → O-aminophenol-β-D-glucoside	animals	(60)
	+ anthronilic acid → β-glucosylated anthronilic acid	plants	(61)

Nucleotide donor	Synthesis observed	Enzyme source	Reference
UDPGalA + $UDPNAcGNH_2$	+ endogenous acceptor → type I specific pneumococcus polysaccharide	bacteria	(48)
UDPG + UDPGal + $UDPNAcGNH_2$	+ endogenous acceptor → type XIV specific pneumococcus polysaccharide	bacteria	(102)
UDPG + UDPGal + UDPGA	+ endogenous acceptor → type VIII specific pneumococcus polysaccharide	bacteria	(49)
$UDPNAcGalNH_2$ + UDPGA	+ oligosaccharides → choindroitin sulphate	animals	(50)
CDP glycerol + UDPG or UDPGal	+ endogenous acceptors → polyglucosyl-glycerol phosphate or polygalactosyl-glycerol phosphate	bacteria	(51)(52)
	<u>Lipids</u>		
UDPG	+ diglyceride → ∝-D-gluco(1-3)diglyceride	bacteria	(53)
UDPGal	+ D-gluco 1-3 diglyceride → ∝-D-gal(1-2) D-gluco(1-3)diglyceride	bacteria	(53)
	+ N-cerebronyl sphingosine → N-cerebronyl-O-D-galactosylsphingosine	animals	(54)

Nucleotide donor	Synthesis observed	Enzyme source	Reference
ADPG	+ maltodextrins or higher MW acceptors depending on source of enzyme → starch	plants	(27)(28)(29)(30)(31)
	+ β (14) glucans → glycogen	bacteria	(32)(33)(34)(35)
$UDPNAcGNH_2$	+ chitodextrins → chitin	fungi	(36)
GDPM	+ endogenous acceptor → mannan	yeast	(103)
CDP ribitol	+ endogenous acceptor → Polyribitol (P)	bacteria	(37)(38)(37a)
CDP glycerol	+ endogenous acceptor → Polyglycerol (P)	bacteria	(39)(38a)
UDPGalA	+ endogenous acceptor → Polygalacturonic acid	plants	(40)
GDPG	+ endogenous acceptor, cello-dextrins increase synthesis → cellulose	plants	(41)
CMP NANA	+ endogenous acceptor → colominic acid	bacteria	(42)
	Heteropolysaccharides		
UDPGA + $UDPNAcGNH_2$	+ unknown acceptor, probably endogenous → hyaluronic acid	animals	(43)
		bacteria	(44)(45)
	+ endogenous acceptor → teichuronic acid	bacteria	(104)
UDPG + UDPGA	+ oligosaccharides → type III specific pneumococcus polysaccharide	bacteria	(46)(47)

TABLE 2: TRANSGLYCOSYLATIONS FROM SUGAR NUCLEOTIDES

Nucleotide donor	Synthesis observed	Enzyme source	Reference
	<u>Disaccharides and oligosaccharides</u>		
UDPG	+ D-glucose-6-Ⓟ → trehalose Ⓟ	yeast animals	(1) (2)
	+ D-fructose → sucrose	plants	(3)(4)(5)
	+ D-fructose-6-Ⓟ → sucrose Ⓟ	plants	(4)(6)(7)
UDPGal	+ D-glucose → lactose	animals	(8)(9)
	+ $NAcGNH_2$ → lactosamine	animals	(8)
	+ sucrose → raffinose	plants	(10)
GDPF	+ lactose → fucosyl lactose	animals	(11)(12)
CMPNANA	+ lactose → sialyl lactose	animals	(13)
UDP xylose	+ oligoxylans → oligoxylans + 1 xylose unit	plants	(14)
	<u>Homopolysaccharides</u>		
UDPG	+ acceptor not known - probably endogenous → β (1-3) Glucan	plants	(14)(15)(16) (17)(18)
	+ cellodextrins → cellulose	bacteria	(19)
	+ oligosaccharide or glycogen) depending on source of enzyme) → glycogen	animals	(20)(21)(22) (23)(24)
		yeast	(25)
		bacteria	(26)

techniques, those sugars found, as yet, only as nucleotide derivatives, such as the diamino sugar from Pneumococcus (Distler, Kaufman and Roseman, 1966), and the extraordinary methylated sugar "vinelose", found in Azotobacter vinelandii (Okuda, Suzuki and Suzuki, 1967), will be found in glycosidic linkages. This may also be the case with the complex oligosaccharide nucleotides found in milk and hen oviduct (Strominger, 1955; Gabriel and Ashwell, 1962; Jourdain, Shimuzu and Roseman, 1961; Takahashi and Suzuki, 1962).

In the synthesis of complex saccharides, the carbohydrate moiety of a nucleotide sugar is transferred to an acceptor, forming a new glycoside. The equilibrium of such a reaction favours the formation of the glycosidic bond, due to the relatively large amounts of energy available from the hydrolysis of the nucleotide phosphate bond (Leloir, Cardini and Cabib, 1960) and there are now many known instances of glycosyl transfer. Table 2 lists some of the transglycosidations observed involving de novo synthesis of a glycosidic compound, whether it be a simple saccharide, or a complex polysaccharide.

The development of cell-free biosynthetic systems dates from the work of Dutton and Storey (1951; 1953), who demonstrated the transfer of a glucuronic acid residue from UDPGA to an aminophenol acceptor, in a liver homogenate (Figure 3), and to date many examples exist of the relatively simple transfer of a sugar moiety from a nucleotide sugar, to a non-glycosidic

acceptor such as a phenol, hydroquinone, or aromatic amine (Table 2). Many of the compounds synthesised are structurally unrelated, the only common feature being the glycosidic moiety. In many instances the function of such compounds is unknown, particularly in plants, some implication in a detoxification mechanism having been found in animals (Boyland, Manson and Orr, 1957).

Classically, the synthesis of trehalose phosphate (Leloir and Cabib, 1953), and sucrose phosphate (Cardini, Leloir and Chiriboga, 1955), followed the demonstration of glucuronosyl transfer, and since then the biosynthesis of other disaccharides and trisaccharides, such as raffinose (Bourne, Walter and Pridham, 1966), and lactose (Watkins and Hassid, 1962; Babad and Hassid, 1966), has been shown to involve glycosidation of a monosaccharide or disaccharide by a sugar nucleotide. Other examples of the transfer of a single sugar unit are common in the field of lipid biosynthesis, and the transfer of L-rhamnose (Burger, Glaser and Burton, 1962), D-glucose (Kaufman et al., 1965), D-galactose (Kaufman et al., 1965; Burton, Sodd and Brady, 1958), and D-mannose (Talamo and Lennarz, 1965), has been shown to occur in the synthesis of glycolipids, in animals and bacteria. The biosynthesis of phospholipids in animals and bacteria is interesting since the transfer of non-glycosidic compounds from cytidine nucleotides has been shown to occur in the biosynthesis of several key compounds. Thus CDP choline has been shown to be a precursor in the formation of lecithin

(Weiss, Smith and Kennedy, 1958) and sphingomyelin (Sribney and Kennedy, 1957), CDP ethanolamine in the formation of phosphatidylethanolamine (Kennedy, 1961), and CDP diglyceride compounds in the formation of phosphatidylinositol, phosphatidylglycerophosphate, and phosphatidylserine (Kanfer and Kennedy, 1964).

De novo synthesis of a polysaccharide was first demonstrated in 1955 (Glaser and Brown, 1955) with the biosynthesis of the simple heteropolysaccharide, hyaluronic acid, from the nucleotide precursors, UDPGA and UDPNAcGNH$_2$. The biosynthetic system was particulate, and it was not clear if an acceptor molecule for the first glycosyl transfer was endogenous, or if one of the sugar nucleotides acted as the first acceptor, perhaps after hydrolysis and liberation of the sugar. If a sugar nucleotide does act as the first acceptor, then either a single nucleotide disaccharide should be formed at the initiation of each chain of hyaluronic acid, followed by stepwise addition of alternate residues of NAcGNH$_2$ and GA, or nucleotide disaccharides may be formed and polymerised into a large molecule. As yet no evidence for nucleotide disaccharides exists, despite a more extensive examination carried out by Markowitz and his group (Markowitz, Cifonelli and Dorfman, 1959; Markowitz and Dorfman, 1962) on a coccus which synthesised hyaluronic acid. Since this first in vitro demonstration of polysaccharide biosynthesis, several homopolysaccharides in plants, animals and bacteria have successfully been synthesised. Table 2 lists some of these examples, such

as glycogen, starch, cellulose and chitin, and also gives examples of the few instances where heteropolysaccharides have been synthesised, notably the complex polysaccharides found in Pneumococcus.

In many instances, the biosynthetic system studied is of a crude, particulate nature, and as such, often any investigation of the system in depth is precluded, but in a few cases the enzyme systems have been sufficiently purified to enable some investigation to be undertaken as to the nature of the acceptor molecule involved in the first glycosyl transfer, the specificity of the enzyme system for the particular nucleotide sugar, or sugars, and the effect of certain compounds of intermediary metabolism on such systems.

There is a considerable body of evidence to suggest that the enzyme systems exhibit considerable specificity towards the nucleotide sugar donors involved in polysaccharide biosynthesis. This is well illustrated by the biosynthesis of glycogen and cellulose, since in bacteria the nucleotide donor in glycogen synthesis is ADPG (Shen and Preiss, 1964; Shen et al., 1966; Greenberg and Preiss, 1965), whereas in animals it is UDPG (Leloir et al., 1959; Leloir and Goldenberg, 1960). In bacteria the donor in cellulose synthesis is UDPG (Glaser, 1958), whereas in plants it is GDPG (Baker, Elbein and Hassid, 1964), although it may be that in some plants UDPG also functions (Drummond and Gibbons, 1965). With the exception of the plant system described by Drummond and Gibbons, all other nucleotide

derivatives of glucose were ineffective or much less effective. There are, however, a few examples of glycosyl transfer in plants from different nucleotide derivatives of the same sugar, as in the system described by Drummond and Gibbons, and in the biosynthesis of rutin (Barker, 1962) where UDP rhamnose and dTDP rhamnose function equally well. There is specificity towards the sugar moiety as well as the nucleotide moiety, and an enzyme system from one type of Pneumococcus has been studied which will transfer glucuronic acid from UDPGA into a polysaccharide, but not galacturonic acid from UDPGalA, whereas the converse is true in an enzyme system from a different type (Mills and Smith, 1962b).

The question of specificity towards the acceptor molecule is the most difficult to investigate, since often the acceptor is endogenous and uncharacterised in the systems employed. Where a system has been solubilised and purified, there may be some information available, as in the glucuronosyl transferase system of liver. The investigators (Isselbacher, Chirabas and Quinn, 1962) were able to separate the enzymic activity into two fractions, one of which catalysed the glucuronosylation of amino groups, and the other of esters, so it may be that the seemingly broadly specific transglucuronosylases of plants and animals (Table 2) are a mixture of enzymes, each with a specific acceptor. In polysaccharide biosynthesis systems, the problem is particularly acute, since it is extremely difficult to separate polysaccharide material from the enzyme

activity. In the purified systems which are available, it is necessary to add acceptors for the initial glycosyl transfer. Such primer molecules are lengthened by stepwise addition of sugar residues to the non-reducing ends (Ginsburg, 1964) as in the addition of xylose from UDPXylose to oligoxylans (Feingold, Neufeld and Hassid, 1960b). Specificity does exist towards the primer as evidenced by the biosynthesis of starch by enzyme systems derived from different sources. Starch synthetase from spinach utilises maltodextrins (Ghosh and Preiss, 1965), whereas that from sweet corn requires a large primer in the form of glycogen, or amylopectin, amylose or starch being ineffective (Frydman and Cardini, 1964), and that from tobacco leaves utilises amylopectin or glycogen, or heated starch granules (Frydman and Cardini, 1960). Many other biosynthetic systems require primers, ranging from low molecular weight oligosaccharides derived from the polymer, to very large polymers of indeterminate molecular weight. The biosynthesis of Pneumococcal polysaccharides is an interesting example of primer requirements following purification of the system, and removal of endogenous acceptors. In the biosynthesis of types I and VIII (Smith and Mills, 1962; Mills and Smith, 1962a; 1962c) there is always residual polysaccharide in the enzyme preparation, and there is no need to add any primer, since endogenous material acts as the initial acceptor, but in type III, all polysaccharide material can be removed by a specific enzyme, and in this case the system requires an oligosaccharide primer

derived from the polysaccharide, of between eight and twelve sugar units (Smith and Mills, 1962). In the light of such experiments, the question arises as to the nature of the very first glycosyl transfer of all in vivo; is a nucleotide sugar the acceptor? Such questions remain unanswered at the moment, although it is known that a single sugar unit can act as the acceptor in the biosynthesis of lactose, where a galactose residue is transferred from UDPGal to a single D-glucose unit (Babab and Hassid, 1966).

The problem of the nucleotide oligosaccharides isolated from milk and hen oviduct must be considered in view of the primer requirements, and pattern of biosynthesis of the systems investigated. It may be that in a few specialised instances, preformed oligosaccharides, attached to nucleotides, may be polymerised to form high molecular weight compounds, but as yet there is no evidence to support this. The compound UDP N-Acetyl neuraminic acid → galactose → $NAcGNH_2$ is of particular interest, since the oligosaccharide is known to be one of the groups in the side chain of two glycoproteins (Spiro, 1962), and it may be that entire groups like this are added to non-glycosidic compounds, preformed. The nucleotides isolated from the hen oviduct lend support to this theory, since the oviduct is a very active glycoprotein synthesising site (Suzuki, 1962; Nomoto and Narashi, 1959), up to 50% by weight of the region being carbohydrate in nature, containing glucosamine, galactosamine, galactose, mannose, sulphate, and phosphate (Nomoto and

Narashi, 1959). In view of the inadequate experimental evidence on this point, one can only speculate, and categorise these nucleotides separately. It seems likely that in all of the systems so far investigated, transfer of single sugar units occurs in a stepwise fashion, illustrated well by the pattern observed in the biosynthesis of chondroitin sulphate (Telser, Robinson and Dorfman, 1966) where the investigators observed that in the formation of this heteropolysaccharide, addition of N-Acetyl glucosamine from $UDPNACGNH_2$ occurred only to oligosaccharides which had glucuronic acid at the non-reducing end, and addition of glucuronic acid from UDPGA only occurred to oligosaccharides with $NACGNH_2$ at the reducing end, ruling out an obligatory disaccharide intermediate of glucuronic acid and N-Acetyl glucosamine, nucleotide or otherwise.

Many of the polysaccharide systems investigated, show marked stimulation by certain molecules of intermediary metabolism. The most striking and best documented example of this is the stimulation of muscle glycogen synthetase by α D-glucose-6-phosphate, but there are other examples in plants and animals, such as the stimulation of hyaluronic acid biosynthesis by N-Acetylglucosamine-6-phosphate (Glaser and Brown, 1955), the stimulation of chitin synthetase by N-Acetylglucosamine(Glaser and Brown, 1957) and the stimulation of β 1-3 glucan synthetase in plants by glycerol or glucose (Thomas and Stanley, 1968) depending on the source of the enzyme. Stimulation of this type would appear to be related to control mechanisms <u>in vivo</u>,

and, in muscle, the effect of G-6-Ⓟ may be quite complex, since it has been shown that two forms of glycogen synthetase exist (Rosell-Perez and Larner, 1964; Rosell-Perez, Villar-Palazi and Larner, 1961), one stimulated by G-6-Ⓟ, the other unaffected. The two forms of glycogen synthetase have been further shown to be interconvertible (Friedman and Larner, 1962) in an energy requiring reaction, and this interconversion is under hormonal control (Craig and Larner, 1964). Precisely how the stimulation of these systems occurs is not clear, since the stimulating compounds are not incorporated into polysaccharide, although it has not been ruled out that they act as intermediate carriers, transferring sugar moiety to acceptor (Ginsburg, 1964). The most likely explanation is that they produce some sort of allosteric configurational change in the active site or sites of the enzymes involved in the systems (Monod, Wyman and Changeux, 1965), this theory being supported by an investigation into the effect of G-6-Ⓟ on yeast glycogen synthetase (Rothman and Cabib, 1967) which suggested that G-6-Ⓟ reversed the allosteric inhibition of glycogen synthetase caused by certain ions. In some plant systems, a mechanism exists, not at the transglycosidase level, but at the preceding step in polysaccharide biosynthesis, that of the sugar nucleotide pyrophosphorylase. In spinach leaf, starch synthetase is not stimulated by a number of phosphorylated intermediates, but ADPG pyrophosphorylase is markedly stimulated by phosphoglyceric acid (PGA) (Ghosh and Preiss, 1965b). Presumably, since PGA

TABLE 3: TRANSGLYCOSYLATIONS FROM SUGAR NUCLEOTIDES

Nucleotide donor	Incorporation of glycosidic moiety observed	Enzyme source	Reference
	Lipids		
CMPNANA	Into sialic acid-free glycolipid	animals	(72)
	Into brain ganglioside	animals	(73)
$UDPNAcGNH_2$	Into brain glycolipid	animals	(72)
UDPGal	Into brain glycolipids	animals	(72)
	Heteropolymers		
UDPG	Into incompletely glucosylated glycoprotein	animals	(74)
UDPGal	Into incompletely galactosylated glycoprotein	animals	(74)
UDPG	Into hydroxymethyl cytosine of phage DNA glucosylated DNA	phage infected	(75)(76)
	gentobiosyl DNA	bacteria	
$UDPNAcGNH_2$	Into incompletely glycosylated glycoprotein	animals	(74)
UDPXylose	Into serine residues of protein	animals	(77)

Nucleotide donor	Incorporation of glycosidic moiety observed	Enzyme source	Reference
CMPKDO			(78)
UDPG			(79)(80)(81)
UDPGal			(80)(81)(82)(88)(89)
$UDPNAcGNH_2$	Into Lipopolysaccharides of Salmonella and Escherichia		(80)(81)
GDPM			(84)(88)
GDP Colitose			(78)
dTDPRh			(84)(85)(88)(89)
CDP Abequose			(86)(87)
UDPNAc muramic acid pentapeptide	Into murein of Gram-positive and Gram-negative bacteria		(92)(93)(94)
$UDPNAcGNH_2$			(96)
UDPG	Into incomplete teichoic acids of Gram-positive bacteria	bacteria	(94)
$UDPNAcGNH_2$		bacteria	(90)

is one of the first compounds formed in CO_2 fixation, the level of PGA present is related to the amount of CO_2 fixation, and corresponding availability of monosaccharides for polysaccharide synthesis. In bacteria the control mechanism also seems to be at the pyrophosphorylase level since, in several different bacteria, glycogen synthetase was not stimulated by glycolytic intermediates, but ADPG pyrophosphorylase was markedly stimulated by a number of compounds, depending on the enzyme system studied (Preiss, Shen and Partridge, 1965; Greenberg and Preiss, 1965; Shen and Preiss, 1965a; 1965b; 1966).

Table 3 lists some other glycosylations observed. In some cases net synthesis of a complete polymer has not yet been achieved, merely incorporation of sugar units from nucleotide sugars into some incomplete polymer acting as an acceptor, or, at best, only a small portion of the molecule has been synthesised. Recently attention has been focussed on mixed polymers of carbohydrate and protein, particularly with regard to the linkages between the different classes of compound. Examples of incorporation into protein are known (McGuire et al., 1965) but the acceptor molecule is difficult to characterise because of its size. In a system incorporating xylose from UDPX into mouse protein, the xylose is found to become linked to the serine residues of protein, and further work is in progress to determine the possibility of a linkage through xylose to chondroitin sulphate (Grebner, Hall and Neufeld, 1966). Analagous studies on a mixed polymer have

been carried out by Kornberg and his group (Kornberg et al., 1959; Kornberg, Zimmerman and Kornberg, 1961) on the formation of glucosylated phage DNA in phage-infected bacteria, showing that glucose from UDPG is incorporated into the hydroxymethyl cytosine residues of DNA.

Table 3 also lists some of the sugar incorporations observed in the synthesis of the extremely complex heteropolysaccharides found in bacteria. In the case of some of the polysaccharides found in the cell envelope of Gram-negative bacteria there may be up to ten monomers present, and several groups of workers have investigated some of these polymers, and obtained for the first time some information on the precise nature of the systems involved in the building up of complex heteropolymers of this type. In general terms, the polysaccharides already discussed, including both homopolysaccharide and heteropolysaccharide types, had not been fully investigated with regard to the precise nature of the enzymic reactions involved, although a few points, such as primer requirements and control mechanisms had been elucidated in some instances.

The synthesis of the homopolysaccharide types theoretically presented no more problem than the synthesis of a simple di- or trisaccharide, since presumably all that is involved is the sequential transfer of activated monosaccharide units to an acceptor or primer. However, the synthesis of a heteropolymer is an interesting theoretical problem. In the case of heteropolymers such as protein or nucleic acid, then of necessity a

FIGURE 9 POSSIBLE MECHANISMS INVOLVED IN THE SYNTHESIS OF A HETEROPOLYMER

REPEATING UNIT $(ABC)_n$

1.

$$\text{ACCEPTOR} + NA \rightarrow {}_{ACC}A + N$$

$${}_{ACC}A + NB \rightarrow {}_{ACC}AB + N$$

$${}_{ACC}AB + NC \rightarrow {}_{ACC}ABC + N$$

2.

$$NA + NB \rightarrow NAB + N$$

$$NAB + NC \rightarrow NABC + N$$

$$NABC + \text{ACCEPTOR} \rightarrow {}_{ACC}ABC + N$$

3.

$$X + NA \longrightarrow XA + N$$

$$XA + NB \rightarrow XAB + N$$

$$XAB + NC \longrightarrow XABC + N$$

$$\text{ACCEPTOR} + XABC \longrightarrow {}_{ACC}ABC + X$$

N ACTIVATING GROUP

X INTERMEDIATE CARRIER

template mechanism must be used to ensure that the correct structure is synthesised, the specificity of the reaction being conferred not by the enzymes, but by the template. It has been proposed (Wilkinson, 1963) that such a mechanism may be operative in the biosynthesis of certain bacterial heteropolysaccharides since early chemical studies appeared to indicate the lack of any well-defined repeating unit in some cases. However, in the light of more recent chemical evidence, it seems likely that all the heteropolysaccharides investigated have, in part at least, a regular repeating structure, and Ginsburg (1964) has argued that, given this, no specific mechanism, other than the specificity of the synthesising enzymes themselves, is necessary to ensure a particular chemical structure. Assuming a repeating unit then several possibilities exist for the biosynthesis of a heteropolymer. Figure 9 illustrates the possibilities for a heteropolymer having the repeating unit ABC. The first possibility is that sequential addition of activated monomer units to an acceptor occurs in much the same way as in the biosynthesis of a homopolymer. The second possibility is that an activated monomer is added to another, to form an activated dimer, followed by addition to form an activated trimer which is then either added to an acceptor, or polymerised and added. The third possibility is that sequential addition of the three monomers occurs to some intermediate carrier, forming a trimer, which is then added to acceptor, or polymerised and added.

A series of very elegant experiments, using both classical chemical techniques and biological techniques, particularly the use of cell-free biosynthetic systems, has been performed by several groups working on different bacterial heteropolysaccharides. Both wild-type organisms and mutants synthesising incomplete heteropolysaccharides have been used, and hitherto unknown structures have been confirmed using an essentially biosynthetic approach. In addition, the use of bacterial mutants readily lends itself to genetic analysis, and many of the genes involved in polysaccharide biosynthesis have been studied. Probably most significant of all, the nature of the biosynthetic systems have been elucidated in some detail, and the indications are that the third possibility illustrated in Figure 9 is operative in several heteropolysaccharide systems in bacteria, involving lipid carriers as an intermediate stage in polymer synthesis.

Any work now published on the biosynthesis of polysaccharides must be viewed in the light of these studies, and the biosynthesis of bacterial polysaccharides will now be considered in detail.

INTRODUCTION

P A R T I I

THE POLYSACCHARIDES OF THE BACTERIAL CELL

Before the early nineteen fifties, virtually nothing was known about the chemical composition or biosynthesis of polysaccharides produced by the bacteria, other than the information available about the extracellular levans and dextrans which had been synthesised *in vitro* a decade earlier. A polymer, similar in its staining reaction with iodine to the animal storage polymer glycogen, had been found as a storage polymer in many species of bacteria, and the principal plant structural polymer, cellulose, was known to occur in the genus *Acetobacter* as an extracellular polymer, but in general terms the structural polymers of bacteria seemed to be very different from those of the algae, fungi and higher plants. In particular, the well known structural polysaccharides of plant cell walls, such as chitin and cellulose, had not been found, apart from cellulose in the genus *Acetobacter*, and since bacteria are physically quite resistant to shock, often maintaining their original shape despite death of the cell, this leads to the conclusion that bacteria must use a structural polymer of entirely different composition.

The principal structure of the bacterial cell responsible for its mechanical rigidity and shape has generally been called

the cell wall, although various other terms such as envelopes, membranes, hulls,and coats have been used. Some authors feel that cell wall implies an inert structure, and prefer other terms to infer possession of functions other than purely mechanical ones. Electron microscope studies of both Gram-positive and Gram-negative bacteria have revealed that whereas the Gram-positive types do appear to have a well defined layer of usually amorphous appearance, quite distinct from the cell membrane, the Gram-negative types often appear to have a many layered structure, sometimes not readily distinguishable from the cell membrane, so perhaps the terminology suggested by Salton (1964) is best applied, cell walls applying to Gram-positives, cell envelopes to Gram-negatives.

Whatever the terminology employed, there is now good evidence to suggest that there is a major chemical component which is responsible for the rigidity of the bacterial cell, and that this material is a complex of amino acids and amino sugars. This new class of structural polymer is found in isolated cell walls and cell envelopes of all the Gram-positive, and most of the Gram-negative bacteria so far examined. Again there is a wealth of descriptive terminology in the literature, the term mucopeptide having been widely employed for the description of these components (Mandelstam and Rogers, 1959), previously more accurately referred to as amino sugar-peptide complexes (Salton, 1952), and Sharon (1963) has suggested peptidopolysaccharides. Lately Weidel and Pelzer (1964) have suggested the name murein

in analogy to the term protein, and this has been further discussed by Martin (1966), making the point that avoiding the term peptide in the designation of the polymer, avoids confusion with the low molecular weight peptides, which arise from the polymer by degradation, and which, as uridine nucleotide derivatives, act as precursors in biosynthesis.

In addition to murein, the cell walls and cell envelopes of bacteria contain other polymers, none of which seem to be necessary for the maintenance of shape and mechanical strength, and the principal difficulty in the study of chemical structure and organisation of a cell wall polymer, is that preparations freed from other polymers are difficult to obtain. Fortunately, in many cases, the investigation of a particular polymer has often been undertaken in an organism in which the polymer is the major component present in the structure, as in the case of the murein component in the cell walls of Gram-positive bacteria. The other important polymers present in both Gram-positive and Gram-negative organisms, presumably carry functions such as the protection of the murein and cytoplasmic components of the cell from attack, by enzymes or chemicals. In Gram-positive organisms, a conspicuous feature of the cell wall may be the presence of a large amount of teichoic acid, a polyol phosphate polymer generally believed to be absent from the Gram-negative bacteria, whereas in the Gram-negative bacteria a unique lipopolysaccharide (LPS) O-antigen complex of protein, lipid, and polysaccharide is present. Such polymers seem

often to be the site of attachment of bacteriophages and may be strongly antigenic. Other accessory wall polymers found in the Gram-positives include a glucose N-acetyl muramic acid polymer in M. lysodiekticus (Perkins, 1963), the group specific polysaccharide antigens of S. pyogenes (Krause and McCarty, 1962) and various rhamnose and amino sugar containing polysaccharides in the genus Lactobacillus (Ikawa, 1961; Baddiley and Buchanan, 1962; Knox and Brandsen, 1962). Bacillus subtilis appears to contain a polymer of N-acetylgalactosamine and glucuronic acid as well as an unusual amino sugar polymer containing a diaminotrideoxyhexose (Janczura, Perkins and Rogers, 1961; Sharon and Jeanloz, 1960).

Of the accessory polymers studied, the teichoic acids of the Gram-positive organisms, and the O-antigenic complexes of the Gram-negatives, have received the most attention, because these compounds constitute important antigenic determinants of the cell, and presumably play an important part in the host-parasite relationship which exists with a pathogenic bacterium. Presumably these compounds are on the outer surface of the cell, and, in particular, the O-antigenic complexes of the Enterobacteriaceae have been studied, because of the demands of medical microbiology for a rapid identification system for this very wide group, comprising a large number of pathogens. The group has been subdivided and classified on the basis of the serological reactivity of the O-antigenic complexes to be found on the surface of every cell. Such studies have shown many

different serological specificities, and conversely many similarities as evidenced by serological cross-reactions, but it has only been comparatively recently that explanations of the serological results have been explained in a chemical sense.

The vast majority of bacteria subdivided by the Gram reaction fall into two broad groups, one group characterised by a cell envelope composed of multilayered components, the inner being a plasma membrane (Gram-negative), the other having a distinct cell wall with a separate cell membrane (Gram-positive). A common feature of both Gram-positive and Gram-negative bacteria is the possession of polysaccharide components, both as storage material in the form of a glycogen-like polymer, and as structural polymers incorporated into the cell wall or envelope. The important aspect of the structure of Gram-positive cell walls is the relative simplicity of the rigid layer which may be almost entirely composed of murein as in Micrococcus lysodiekticus (Perkins and Rogers, 1959). The ease with which almost pure preparations of murein can be obtained by the technique described by Salton and Horne (1951) for the isolation of cell walls of bacteria, based on the rigidity of such structures and their resistance to mechanical shock, is responsible for the great increase in knowledge of this structural polymer in the last fifteen years. The more complex envelopes of Gram-negative organisms also possess murein, but there is little doubt that in contrast to the Gram-positive bacteria, the bulk of the envelope is made up of

protein, lipid, and polysaccharide complexes. Luderitz, Jann and Wheat (1968) in their comprehensive review of the polysaccharides of Gram-negative bacteria, have considered that the plasma membrane of the cell is surrounded by a layer composed of murein (0.1 - 10% cell dry weight) which is enveloped by an interdigitated lipophilic complex of lipopolysaccharide (1 - 5% dry cell weight), phospholipid, and protein.

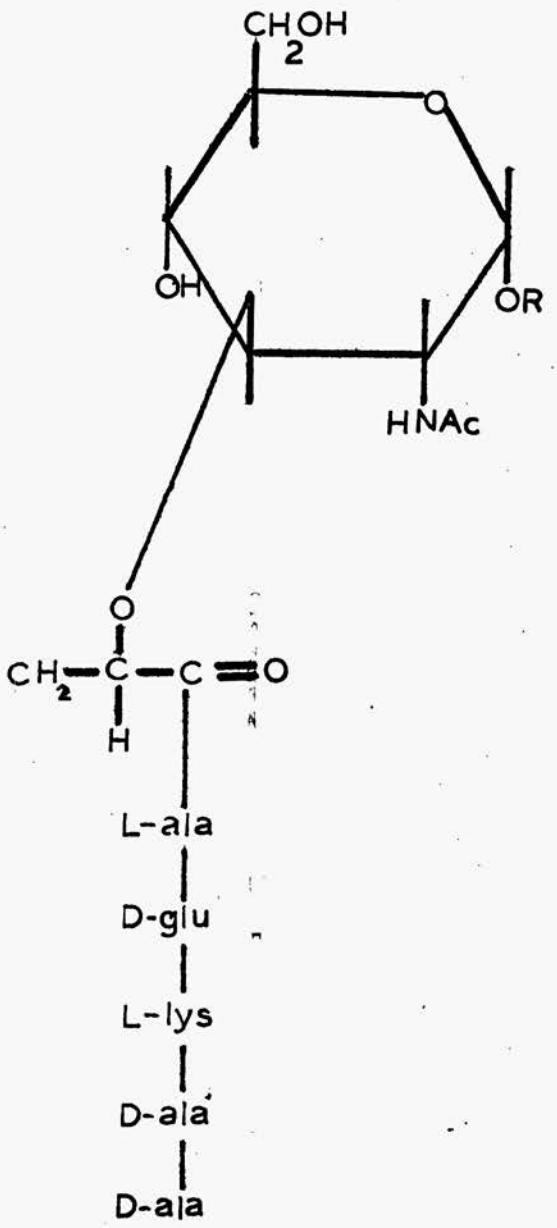
A further feature common to both Gram-positive and Gram-negative bacteria is the frequent possession of polysaccharide material, generally hydrophilic in nature, on the cell surface. This may surround the cell in the form of a capsule (often termed capsular or K antigen) or it may be found in the form of an amorphous slime, not affixed to the cell surface, simply dissolved in the culture medium. Slime is also produced by encapsulated cells, so there can be no definite distinction between capsular material and slime material (Wilkinson, 1958). In general these extracellular polysaccharides are only loosely linked to the cell surface, primarily by physical bonds, and can usually be easily extracted with water (Luderitz, Jann and Wheat, 1968) whereas the components of the cell wall or envelope are attached in a more complex fashion, either covalently, or as ligands, and as such require more drastic treatment to release them from other polymers.

Remarkable variation exists within the bacterial cell with regard to polysaccharides synthesised, ranging from the simple homopolymeric type such as glycogen, to the extremely complex

heteropolymeric types such as murein, LPS and teichoic acid. Each of the heteropolymers is interesting for different reasons. Murein, despite having only two sugar components, is extensively modified by the addition of amino acids, and precisely how and when these are added is of significance in any consideration of mixed polymer synthesis. LPS is extraordinary in the number of monomers involved in the overall structure, and the polysaccharide portion of the molecule consists of three distinct regions, a remarkable problem in synthesis and control for the cell. Teichoic acid is interesting in that, whereas it is not strictly a polysaccharide, the method of synthesis appears to be similar, and other components such as amino acids and sugars are added at some point in the biosynthesis.

There is now a plethora of information available about the structure and biosynthesis of these compounds and they will be considered individually.

FIGURE 10 UDPNAcMurA PENTAPEPTIDE



MUREIN

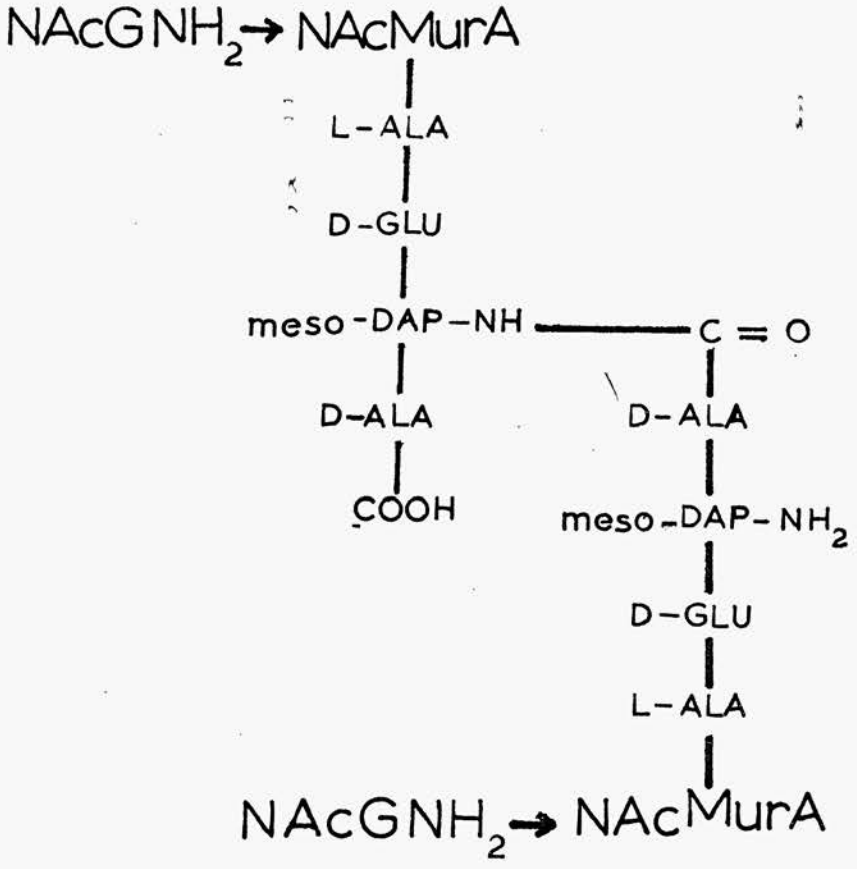
The cell walls of Gram-positive bacteria so far examined have revealed a strikingly similar and relatively simple composition. The few compounds that are regularly found in these walls are believed to be components of a structure which is the basic structural polymer, unique to the bacterial world. These components are N-acetyl glucosamine, a lactic acid ether of N-acetyl glucosamine which is commonly called N-acetyl muramic acid, and the amino acids L and D alanine, D glutamic acid, and either L lysine or diamino pimelic acid in the meso form.

The earliest and most significant indication of the structure of this structural polymer, variously called muco-peptide, amino sugar peptide complex, glucosaminopeptide complex, and murein (Martin, 1966), comes from the study of the now famous nucleotides isolated by Park (1952) on treatment of an S. aureus strain with penicillin. These nucleotides are a mixture of UDP N-acetyl muramic acid (UDPNACMurA) derivatives, some of which have a complete set of the typical murein amino acids attached to the carboxyl group of N-acetyl muramic acid in the form of a pentapeptide chain (Figure 10) (Park and Strominger, 1957), the sequence and configuration of the amino acids having been elucidated by analysis of the products of partial acid hydrolysis, and confirmed later by biosynthesis of the UDP sugar pentapeptide by stepwise addition of the given

amino acids to a UDP acceptor molecule with the preceding amino acid in the C terminal position (Ito and Strominger, 1960a; 1960b). Quite early the qualitative and quantitative parallels in the composition of this UDPNAcMurA pentapeptide and bacterial cell walls, and the hypothesis was advanced that this compound served as a precursor for murein synthesis, the system somehow being blocked by the addition of penicillin (Park and Strominger, 1957).

Other relevant information comes from the isolation and identification of fragments obtained by the specific action of different hydrolysing enzymes, in particular egg-white lyzozyme. In S. aureus and M. lysodiekticus, despite the large amount of murein present in the cell wall, only very small amounts of low molecular weight compounds are released on lyzozyme treatment, suggesting that the peptide chains attached to NAcMurA are extensively cross-linked (Mandelstam and Strominger, 1961; Salton, 1957). Despite this, using lyzozyme, which acts as a N-acetylmuramidase, several groups of workers characterised small amounts of low molecular weight substances from S. aureus as NAc GNH_2 1⟶6 NAc MurA disaccharides, some with peptide chains attached (Salton, 1956; Berger and Weiser, 1957; Salton and Ghuysen, 1960). Later, following a reappraisal of the identification techniques, and confirmatory work on M. lysodiekticus as well as S. aureus, the linkage was shown to be β 1⟶4, and it seemed likely that the repeating unit of the polymer was ⟶4 $NAcGNH_2$ 1 $\overset{\beta}{\longrightarrow}$ 4 NAcMurA 1— (Park and

FIGURE 11 FRAGMENT OBTAINED BY LYZOZYME DIGESTION OF *E. coli* MUREIN



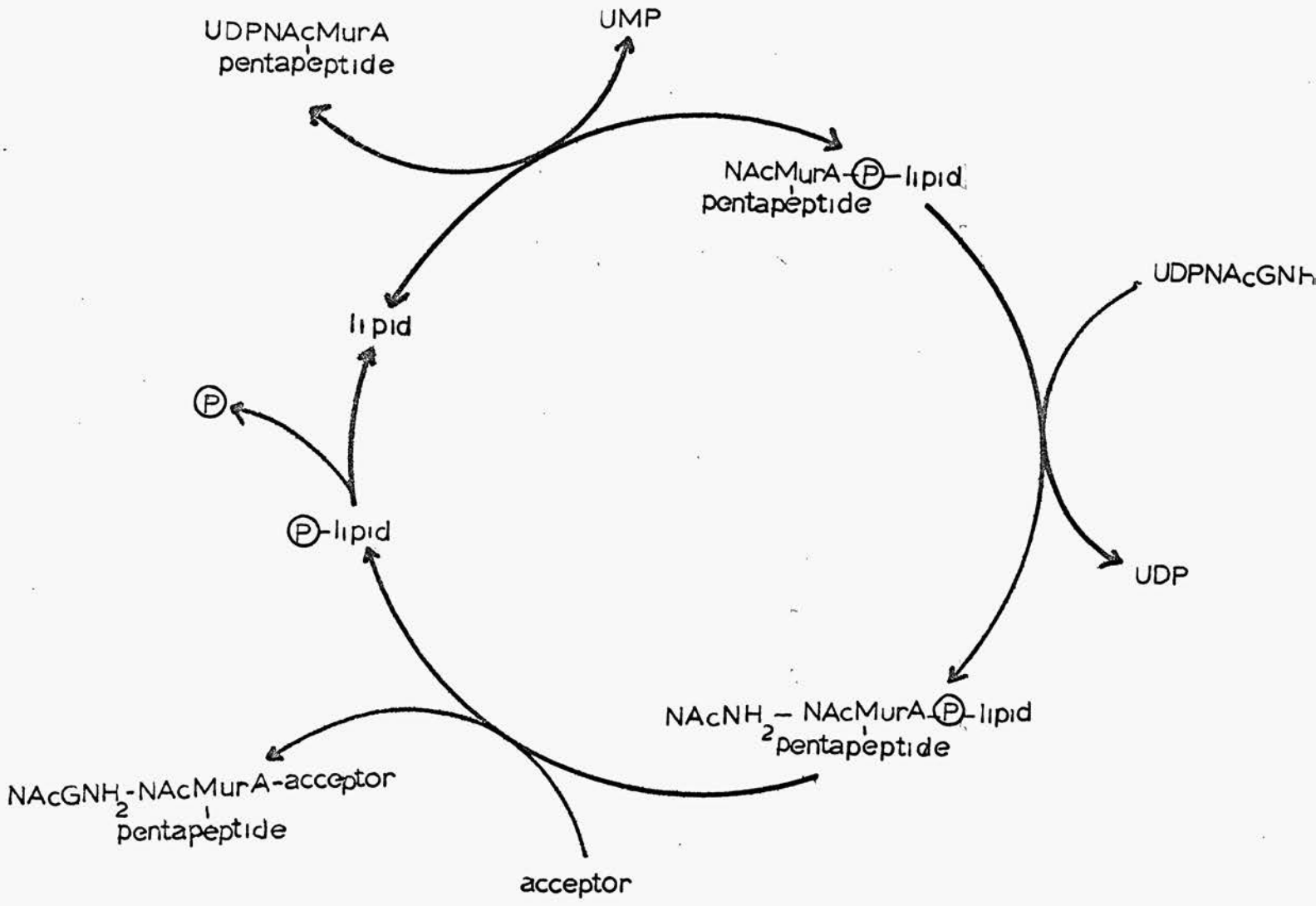
Griffith, 1962; 1964; Ghuysen and Strominger, 1963; Tipper, Ghuysen and Strominger, 1965). Gram-negative organisms have been particularly useful in the elucidation of murein structure, despite the relatively small amounts of murein present in the cell envelope, due to the relative absence of extensive cross-linking. In 1958 Weidel and Primosigh had observed that isolated Gram-negative cell walls tended to lose their shape and rigidity when treated with lyzozyme, releasing small molecular weight fragments containing characteristic murein components. These and later similar observations on special groups such as the acid fast bacteria (Takeya and Hisatsune, 1963a; 1963b) had been responsible for the concept of murein as a structural polymer common to all bacterial cells. Pelzer (1962; 1963a; 1963b) followed up these reports, and reported that lyzozyme split the murein component of E. coli envelopes completely into a few small molecular weight components. The structure of some of these components was elucidated by the use of specific cleavage enzymes obtained from E. coli cells, and one of the larger compounds is illustrated in Figure 11, showing two disaccharide repeating units held together by cross-linking of meso diaminopimelic acid in one chain to the terminal alanine in an adjacent chain. On the basis of these results, and those obtained from the lyzozyme treatment of S. aureus and M. lysodrekticus, it seems certain that all the necessary properties are available for the construction of a two or three dimensional network (Martin, 1966).

In contrast to the sugar moieties of the polymer, the peptide moieties are not so well understood. Again the investigation of sugar nucleotides has proved to be of value. The Park nucleotides (Park, 1952a; 1952b; 1952c) and those accumulated by M. lysodiekticus on treatment with a uracil analogue (Roger and Perkins, 1960; Czerkawski, Perkins and Rogers, 1963) contain glutamic acid, lysine and alanine in the ratio 1:1:3, the same as those accumulated by E. coli cells on penicillin treatment, with the exception that DAP replaces lysine as with other Gram-negative bacteria (Strominger, Scott and Threnn, 1958; Plapp and Kandler, 1965). Diamino acids such as lysine or DAP are clearly important by virtue of their ability to participate in more than two peptide bonds. Other diamino acids have been found replacing DAP or lysine in a few recorded instances such as L-ornithine in M. radiodurans (Work, 1964) and 2-4 diaminobutyric acid in Corynebacteria spp. (Perkins and Cummins, 1964). In E. coli the degree of cross-linking is 30% (Martin, 1963), the linkage involving a simple bridge between an alanine residue in one chain and the DAP residue in an adjacent chain (Pelzer, 1963b), but in Gram-positive organisms the situation is more complex and the cross-linking more complete, and may involve other amino acids. Glycine is to be found in the cell walls of S. aureus in large amounts, and in M. lysodiekticus in smaller amounts. It has been considered to be involved in cross-linkage of murein peptide chains, but has never been found on a nucleotide

derivative (Salton, 1961). Another striking feature to emerge from the comparison of penicillin induced nucleotides and fragments obtained by murein digestion was the difference in the number of alanine residues in the compounds. The fragments obtained by Pelzer (1962a; 1963a; 1963b) from E. coli have the amino acids, glutamic acid, DAP, and alanine, in the ratio 1:1:2, and the use of a combination of murein hydrolysing enzymes has revealed a similar picture in M. lysodiekticus, lysine replacing DAP (Ghuysen, 1960; 1961). These analogies were not to be resolved until a series of elegant in vitro cell-free biosynthetic studies were carried out, mainly by the Strominger group.

Much research has been devoted to cell-free biosynthetic systems employing Park nucleotides. The synthesis of the most complex of these, the UDPNAcMurA pentapeptide, has already been described (Ito and Strominger, 1960a; 1960b), the normal pathways of protein biosynthesis not being operative in this case, since the reactions are not sensitive to ribonuclease, indicating that the amino acids are not activated through the mediation of transfer ribonucleic acid (sRNA) (Hancock and Park, 1958). Cell-free systems from S. aureus have been described which incorporate UDPMurA pentapeptides and UDPNAcGNH$_2$ into murein-like material (Chatterjee and Park, 1964; Meadow, Anderson and Strominger, 1964). The murein synthetase is found specifically associated with particulate cell fragments which consist largely of ruptured cell membranes, and little

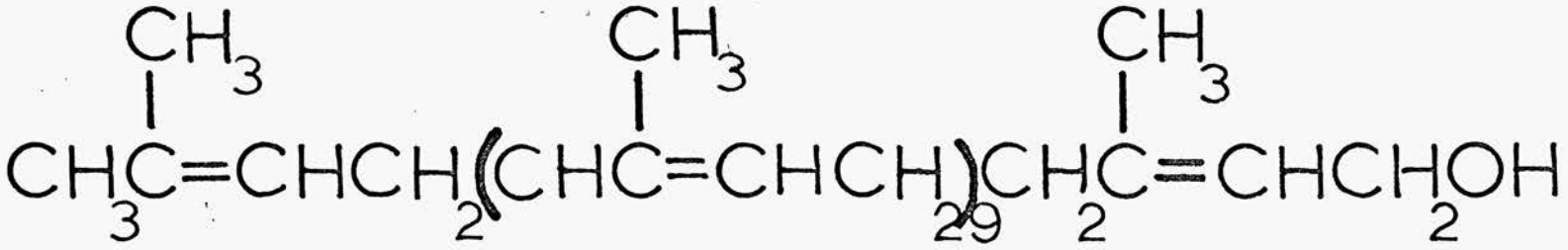
FIGURE 12 ROLE OF LIPID-LINKED INTERMEDIATES IN MUREIN SYNTHESIS



incorporation of UDPNAcMurA penta. or UDPNAcGNH$_2$ was observed in the absence of the other. The product described by Meadow et al. was soluble by digestion with lyzozyme, but it was clear that it was not identical with naturally occurring murein from this organism, since the peptide chain showed no cross-linkage, and contained the extra alanine residue. The real significance of the results of Meadow et al. was the observation that UMP appeared to be the product formed from UDPNAcMurA pentapeptide on incorporation, contrary to the belief that a nucleoside diphosphate was the product formed when transfer of a sugar from a nucleoside diphosphate sugar to a polymer took place. This result was confirmed by Struve and Neuhaus (1965) leading to the hypothesis that instead of direct incorporation of NAcMurA pentapeptide into the polymer, the first step was the incorporation of NAcMurA pentapeptide phosphate into an intermediate acceptor molecule.

Further work by Strominger and his group (Anderson et al., 1965) was to clarify the nature of this acceptor. Particulate preparations from S. aureus and M. lysodiekticus catalyse the stepwise transfer of NAcMurA pentapeptide phosphate followed by NAcGNH$_2$ to a lipid carrier. The scheme proposed by Anderson et al. is shown in Figure 12. NAcMurA pentapeptide phosphate is transferred to lipid, releasing UMP, followed by transfer of NAcGNH$_2$ releasing UDP. This compound seems to be the immediate murein precursor, suitable for membrane penetration, and the disaccharide pentapeptide is transferred to an acceptor

FIGURE 13 STRUCTURE OF LIPID INVOLVED IN MUREIN SYNTHESIS



which is presumably the growing chain, liberating a phosphorylated lipid carrier, which is regenerated, inorganic phosphate being formed. Some confirmation for the reaction was obtained by the observation that the inorganic phosphate liberated was derived from UD^{32}PNAcMurA pentapeptide, and both the monosaccharide and disaccharide intermediates have been obtained, the former by omitting UDPNAcGNH$_2$ from the reaction mixture. The lipid intermediates have been extracted and purified (Anderson and Strominger, 1965; Dietrich, Colucci and Strominger, 1967; Higachi, Strominger and Sweeley, 1967), decomposition studies and mass spectrometry studies have indicated that the lipid is a derivative of a C_{55} isoprenoid alcohol, linked to the sugars by a pyrophosphate bond (Figure 13). Lipid intermediates of this type derived from M. lysodrekticus have been found to be active in cell-free systems derived from M. roseus and S. epidermiditis, indicating the similarities of such systems in different bacteria.

The cell-free biosynthetic system was also to resolve the problem of the extra alanine residue found in the precursors, and the method of cross-linking of the peptide chain. Wise and Park (1965) had suggested that since, in the presence of penicillin, there is increased incorporation of alanine into the cell wall of S. aureus, associated with an increase in the number of N terminal glycine residues, the final step in cell wall biosynthesis is cross-linking of the chains by glycine residues in conjunction with liberation of the terminal alanine

residue, and that this step is sensitive to penicillin. Further work on the biosynthetic system in S. aureus (Matsuhashi, Dietrich and Strominger, 1965; Anderson et al., 1967) was to establish that pentaglycine chains were formed and bound to the ϵ group of lysine while the disaccharide pentapeptide was bound to the lipid carrier, the formation of the glycine chain requiring the mediation of sRNA in contrast to the pentapeptide chain. Similar results were achieved with M. lysodiekticus where a single glycine residue is involved (Katz et al., 1967), M. roseus where threonyl sRNA participates, and S. epidermitis which has serine cross bridges (Strominger, Izaki and Matsuhashi, 1967). The final transpeptidation, involving linkage to the alanine residue of another chain, with concomitant liberation of the terminal alanine residue from the chain, has not yet been demonstrated in a cell-free system derived from a Gram-positive organism, but this reaction has been shown to occur in an E. coli system, where simpler cross-linking occurs (Araki et al., 1966). Cross-linking of the DAP residue in one chain was observed to occur with the alanine residue second from the end of an adjacent chain, the terminal residue being split off, presumably to provide energy for the reaction. The reaction was shown to be inhibited by penicillin, confirming the hypothesis of Wise and Park (Izaki, Matsuhashi and Strominger, 1966).

Clearly, the mechanism of synthesis of the 'murein sacculus' or the 'bag-shaped macromolecule' described by

Weidel and Pelzer (1964) in their review of the subject, is at hand.

TEICHOIC ACIDS

Following the isolation of CDP ribitol and CDP glycerol from Lactobacillus arabinosus, and the conformation of their structures by chemical synthesis (Baddiley, Buchanan and Fawcett, 1959), the probable role of these compounds in polymer synthesis was predicted. The biosynthetic functions of these compounds became almost certain, when polymers of ribitol phosphate and glycerol phosphate were isolated from trichloracetic acid extracts of L. arabinosus (Armstrong et al., 1958) and other bacteria. The localisation of these compounds in Gram-positive cell walls led to the description of this new class of polyol phosphate substances, as the teichoic acids (Armstrong et al., 1958). These compounds do not appear to have a role in maintaining the structural rigidity of the cell, since it has been shown that removal from the cell wall leaves a rigid structure, the murein component maintaining the integrity of shape (Archibald et al., 1961).

Whereas it has been generally inferred that only Gram-positive organisms possess teichoic acids, and certainly more Gram-positive organisms have been tested (Baddiley, 1961), Lilly (1962) has reported the presence of a polymer containing ribitol, phosphate, glucose and murein from an E. coli strain, suggesting a teichoic acid type of compound, linked to murein in some way. Further evidence for this was the observation that CDP ribitol and CDP glycerol accumulated in the growth

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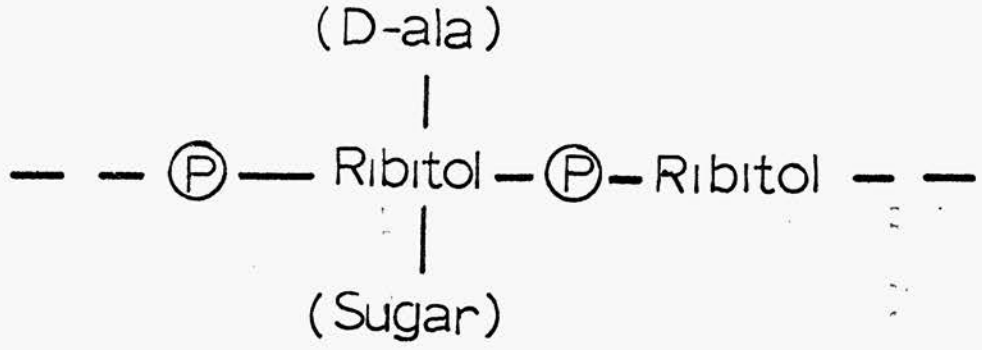
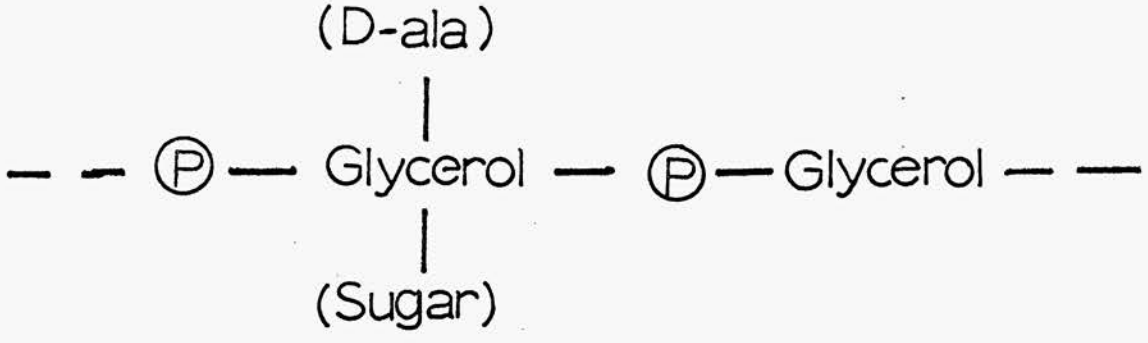
medium, but this investigation has never been followed up, and to date there is no unequivocal evidence for the presence of teichoic acids in Gram-negative organisms. The only other sources of compounds related to the teichoic acids are the extracellular polysaccharides of the Pneumococcus. Rebers and Heidelberger (1959) reported ribitol phosphate in a pneumococcal polysaccharide, and since then a number of such polysaccharides have been examined, and the occurrence of both ribitol phosphate and glycerol phosphate recorded in a number of instances (Shabarova, Buchanan and Baddiley, 1962).

Gram-positive cell walls usually contain either the glycerol or the ribitol polymer (Armstrong et al., 1959; Baddiley, 1961) but, in some instances, both polymers are present (Armstrong et al., 1959; Armstrong et al., 1958). Following the detection of teichoic acids within the cell wall, a teichoic acid, generally of the glycerol type, was found to be present in an intracellular form (Baddiley, 1961), and a further investigation has confirmed this result, determining the localisation and type of teichoic acid in many different species of bacteria (Baddiley and Davidson, 1961).

The function of the cell wall and intracellular teichoic acids has yet to be determined, since they are not structural. It has been shown that they constitute important antigenic components of the cell, and are presumably on the outer cell surface, presumably playing an important role in the interaction with the environment (Sanderson, Juergens and Strominger,

FIGURE 14 DIAGRAMMATIC REPRESENTATION OF THE TWO TYPES OF TEICHOIC ACID





1961).

From the studies of Armstrong et al. (1958; 1959) and other workers such as Keleman and Baddiley (1961) there appears to be two general types of teichoic acid (Figure 14), a common feature being the presence of ester-linked D-alanine. Other constituents such as sugar components are common, depending on the source of the teichoic acid, thus the repeating unit of S. aureus cell wall teichoic acid is 4-O-D N-Acetylglucosamine D-ribitol 5-Ⓟ, the ribitol units being esterified by D-alanine (Ishimoto and Strominger, 1967).

Precisely how the cell wall teichoic acids are attached to other cell wall polymers is disputed. S. aureus cell walls on treatment with lyzozyme do not release small molecular weight fragments, probably due to O-acetyl groups on the sugar constituents of murein (Brumfitt, Wardlaw and Park, 1958) and the cross-linking of the peptide chains, but also probably because of additional linkages due to teichoic acid, which may account for up to 60% of the cell wall structure (Salton and Pavlik, 1960). Mandelstam and Strominger (1961) have considered that peptide linkages between teichoic acid alanine residues and murein peptides might be possible. Covalent linkage between the two polymers has had most support and Strominger and Ghuysen (1961) have obtained fragments of murein with teichoic acid attached, using a murein hydrolysing enzyme, but the nature of the attachment was not determined. However, similar results have recently been obtained using new enzyme

systems (Ghuysen, Leyh-Bouille and Dierickx, 1962; Hash, 1963) and a covalently linked complex of murein and teichoic acid has been obtained from S. aureus (Ghuysen et al., 1965). A disaccharide of $NAcGNH_2$ and 6-O diacetyl MurA carried a teichoic acid chain attached through a phosphodiester linkage between ribitol at the 5 position, and one of the sugars was isolated. The chain length has yet to be determined, and there are conflicting reports as to the chain length of teichoic acids in cell walls. Ghuysen, Tipper and Strominger (1965) consider 50-40 ribitol units in S. aureus, but this view is not shared by Baddiley and his group (Haus et al., 1965) who suggest 8, but it may be that different strains vary considerably in chain length.

The biosynthesis of polyribitol phosphate from CDP ribitol was first demonstrated by Glaser (1963a; 1963b) using a particulate preparation from L. plantarum, and the synthesis of polyglycerol phosphate has also been reported, preparations from B. licheniformis and B. subtilus catalysing the formation of a chain of about thirty units (Burger and Glaser, 1964). The precise mechanism of polymerisation has not yet been elucidated in these systems, although Baddiley (1968) has indicated that his group have obtained evidence, as yet unpublished, that a phospholipid intermediate stage, similar to that in murein biosynthesis, may be involved.

Since the naturally occurring teichoic acids have a variety of substituents such as sugars and D-alanine in

addition to the polyol phosphate chain, it is interesting to speculate as to when these components are incorporated. As yet no results have been published on the incorporation of the alanine residue, but several workers have examined the incorporation of the sugar residues. The teichoic acid of S. aureus is of the ribitol type, different strains being characterised by different ratios of α and β linked $NAcGNH_2$ (Baddiley et al., 1962a; 1962b; Sanderson, Strominger and Nathenson, 1962). Particulate preparations would incorporate $NAcGNH_2$ from the UDP derivative into a ribitol teichoic acid which had been enzymically treated to remove $NAcGNH_2$, intact teichoic acid being ineffective (Nathenson and Strominger, 1962; 1963). Both α and β linkages were formed in the ratio found in the cell wall of the organism, but it was not determined whether more than one enzyme was responsible. Similar results have been shown in B. subtilis preparations, which incorporate glucose from UDPG into an incompletely glucosylated glycerol polymer (Glaser and Burger, 1964). Further studies have shown that probably the sugar unit is incorporated into the growing polyol chain since Ishimoto and Strominger (1967) have shown that $NAcGNH_2$ was incorporated much more efficiently into polymers in conjunction with the synthesis of the ribitol polymer. It has also been shown that in several strains of S. aureus the linkages of $NAcGNH_2$ incorporated into the growing chain were substantially the same as in the polymer extractable from the cell wall, although the systems studied in all cases had the

ability to synthesise both α and β linkages, an interesting example of enzyme specificity (Nathenson et al., 1967). In all cases there was no means of establishing if new chains had been initiated, or old ones finished, and the amount of synthesis observed depended on the amount of particles present in the enzyme system, perhaps indicating that murein could be the initial acceptor, since no free phosphomonoester groups were detected (Nathenson et al., 1967).

A new type of teichoic acid polymer was first synthesised in cell-free preparations derived from B. licheniformis (Burger and Glaser, 1966). Preparations catalyse the incorporation of galactose or glucose from UDP derivatives into a growing poly-glycerol phosphate polymer, incorporation of either glycerol or the hexose being maximal in the presence of the other. The product was shown to contain the repeating unit glycerol-Ⓟ-glucosyl or glycerol-Ⓟ-galactosyl , the two polymers apparently being synthesised independently. The synthesis of the polymers induced the workers to examine the cell walls of the organism, where such polymers were detected. The occurrence of such polymers and their relationship to other teichoic acids remains to be investigated.

LIPOPOLYSACCHARIDES

The cell envelope of many Gram-negative bacteria contains a complex high molecular weight polymer, which is immunologically specific, possesses endotoxic activity, and may have phage receptor sites. This heteropolymer appears to overlie the murein layer and may account for the difference in sensitivity of Gram-positive and Gram-negative organisms to attack by lyzozyme. Interest in such compounds developed originally through immunological systems related to medical problems, so it is not surprising that members of the largely pathogenic Salmonella, Shigella and Escherichia group have been most studied, and a system of classification and identification based on the immunologic properties of these heteropolymers or O or somatic antigens as they have come to be known.

It has been shown that extraction of O-antigens under extremely mild conditions yields a complex consisting of protein, lipid, and a polysaccharide polymer with a tightly bound lipid component (lipopolysaccharide or LPS) (Boivin and Mesrobeanu, 1935; Morgan, 1937), and it may be that O-antigens exist in the cell in this form. As a result of early work by Boivin and his group (Boivin and Mesrobeanu, 1933a; 1933b; 1933c; 1933d; 1933e; 1935; 1936; 1937) it became clear that the antigenic properties of the O antigen were associated with LPS, and that the polysaccharide part which can be liberated from the complex by acid hydrolysis, carried the complete O antigenic

specificity of the cell, the basis for the Kauffmann scheme of classification (Kauffmann, 1961). Despite carrying the serological specificity, the LPS portion is only a weak antigen, but can be made strongly antigenic by re-association with the protein component. The lipid part of the LPS, so called lipid A, seems to be responsible in part for the toxic properties of the O-antigen complex, since it has been shown (Binkley, Goebel and Perlman, 1945) that the protein component of the complex can be obtained in two forms, one form being non-toxic, the other, believed to be conjugated to lipid A, being highly toxic. Furthermore, Morgan and Partridge (1941) have shown that conjugated protein can be recombined with LPS to give a complex, antigenically and endotoxically similar to native O antigen. The protein components of many Gram-negative O-antigens seem to be quite similar, since conjugated protein from one strain can be combined with LPS from another, to give O-antigenic complexes with the serological specificity of the LPS portion (Morgan and Partridge, 1941).

The other lipid component extractable, designated lipid B, is only loosely linked to the whole antigen complex, from which it can easily be dissociated (Binkley, Goebel and Perlman, 1945; Morgan and Partridge, 1941), its precise function remaining unclear, since it does not appear to be essential for the endotoxic and serological properties of the complex. Analysis has shown that it is predominantly phospholipid in nature (Kaneshiro and Marr, 1962) and very recently fresh interest has been

engendered by the observation that a lipid B-like material participated in the biosynthesis of the polysaccharide portion of the complex (Rothfield and Horecker, 1964), perhaps by altering the spacial configuration of the polysaccharide molecule (Rothfield and Takeshito, 1965; 1966).

Lipopolysaccharides possess many interesting biological properties, and have therefore attracted the attention of large numbers of workers, particularly since the polysaccharide portion of the molecule carries serological specificity, and an extensive investigation of the serological specificities of the Enterobacteriaceae has been undertaken (Kauffmann, 1961), affording a unique chance to correlate serological activity and cross reaction with chemical structure. The other significant aspect of such an investigation is the ready availability of genetic systems within the Enterobacteriaceae, enabling a remarkable three-sided approach to the problem to be used.

Most investigators have turned their attention to the polysaccharide portion of the molecule, and the lipid A portion has not been extensively studied. The presence of such a lipid has been demonstrated in many laboratories (Tal and Goebel, 1950; Westphal and Lüderitz, 1954; Shear et al., 1943) but only fragmentary analyses have been performed. Lipid A preparations examined fairly recently seem to be similar in many Gram-negative bacteria (Lüderitz, Jann and Wheat, 1968) and recent results seem to indicate that the constituents include β-hydroxymyristic acid, glucosamine, and phosphate (Westphal

and Lüderitz, 1954; Burton and Carter, 1964) and it has been suggested from the available data that lipid A is composed of a backbone of N- β hydroxymyristyl glucosamine phosphate, in which all the available groups of the glucosamine residues, as well as of the hydroxymyristic acids, are esterified by long chain fatty acids, and possibly acetyl groups (Lüderitz, Jann and Wheat, 1968). The lipid is covalently linked to polysaccharide, since the LPS can be extracted by a variety of solvents, the polysaccharide portion being split off by acid hydrolysis (Davies, 1955; Sutherland, Lüderitz and Westphal, 1965) but how the LPS is attached to the other lipid and protein portion of the O-antigenic complex is not known, although Wheat (1964) has suggested that the primary attachment is via physical bonds.

While the protein and lipid components of the O antigens appear to be largely chemically similar, the polysaccharide components vary greatly with respect to sugar composition and structure, in parallel with the very wide range of serological specificities encountered. More than twenty different sugars have been found in such polysaccharides, some sugars being commonly found, such as heptose, 2 keto 3 deoxyoctulosonic acid (KDO), glucose, galactose, others being more rare, such as 6 deoxyhexoses, 3,6 dideoxyhexoses, hexosamines, and pentoses, and occasionally uronic acids. In the Salmonellae in particular, tables have been drawn up relating constituent sugars with serological groups, and work has now resolved the correlation

between structural units of the polysaccharide and immunologically determinant groups (Lüderitz, Jann and Wheat, 1968).

In the Salmonellae the serological specificities of the wild type smooth organisms are extremely numerous, and corresponding chemical diversity immense, and our present day knowledge of chemical structure stems largely from intuitive studies of mutant organisms which have arisen during laboratory culture, so called rough R types which have altered surface structure, and are distinguishable from the wild type on agar plates. On the basis of the chemical examination of several mutants it has been determined that the polysaccharide moiety of O antigenic smooth strains is composed of two distinct regions, which differ chemically and immunologically. These are a core which contains certain 'basal' sugars, heptose, KDO, glucose, galactose, and $NAcGNH_2$, and an outer region made up of repeating tri- or tetrasaccharide units which carry the immunological specificity of O-antigenicity, certain immunologically important sugars such as dideoxyhexoses and deoxyhexoses being present. In its simplest form the suggestion is that different Salmonella O serotypes differ only in the specific side chains, which are attached to a common basal structure.

Most information was derived from the study of two R mutants which were allocated to the serological groups RI and RII. In contrast to the wide variety of smooth serotypes known, many strains mutated to give RI or RII types, which seemed to possess LPS of the same chemotype, the RI type

possessing the sugars heptose, glucose and galactose, and the RII an additional $NAcGNH_2$, O specific sugars being entirely absent (Lüderitz et al., 1960; Kauffmann et al., 1961). The common structure possessed by these mutants derived from many different O-antigenic types, suggested to Lüderitz and Kauffmann and their co-workers that a basic core, probably similar in composition to the LPS of the RII mutants, did exist in all O-antigenic types, the RI type being slightly less complete, lacking one of the basal sugars (Lüderitz et al., 1960; Kauff- et al., 1961).

Further confirmation and structural detail followed on the investigation of other R types isolated in several laboratories. In particular a mutant of S. typhimurium which lacked the enzyme UDPG pyrophosphorylase, was found to have a LPS containing only heptose and phosphate, being entirely devoid of O antigenicity (Fukasawa, Jokura and Kurahashi, 1962; Sundarajan, Rapin and Kalckar, 1962) suggesting that glucose must be the linkage point between heptose and other core sugars. Another mutant deficient in the enzyme UDPG-4- Epimerase was found to have a LPS containing heptose, phosphate, and glucose, and another sugar characterised as 2 keto 3 deoxyoctonic acid (KDO) (Osborn, 1963). This strange sugar KDO had previously been characterised as a component of the LPS of an E. coli serotype (Heath and (Ghalambor, 1963) and on the basis of its presence in the incomplete polysaccharide of the UDPG-4-Epimeraseless mutants, and its presence in other mutants investigated, Osborn (1963)

FIGURE 15 CORE STRUCTURE OF *Salmonella* LPS

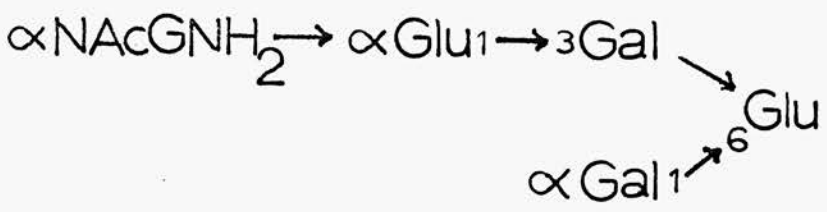
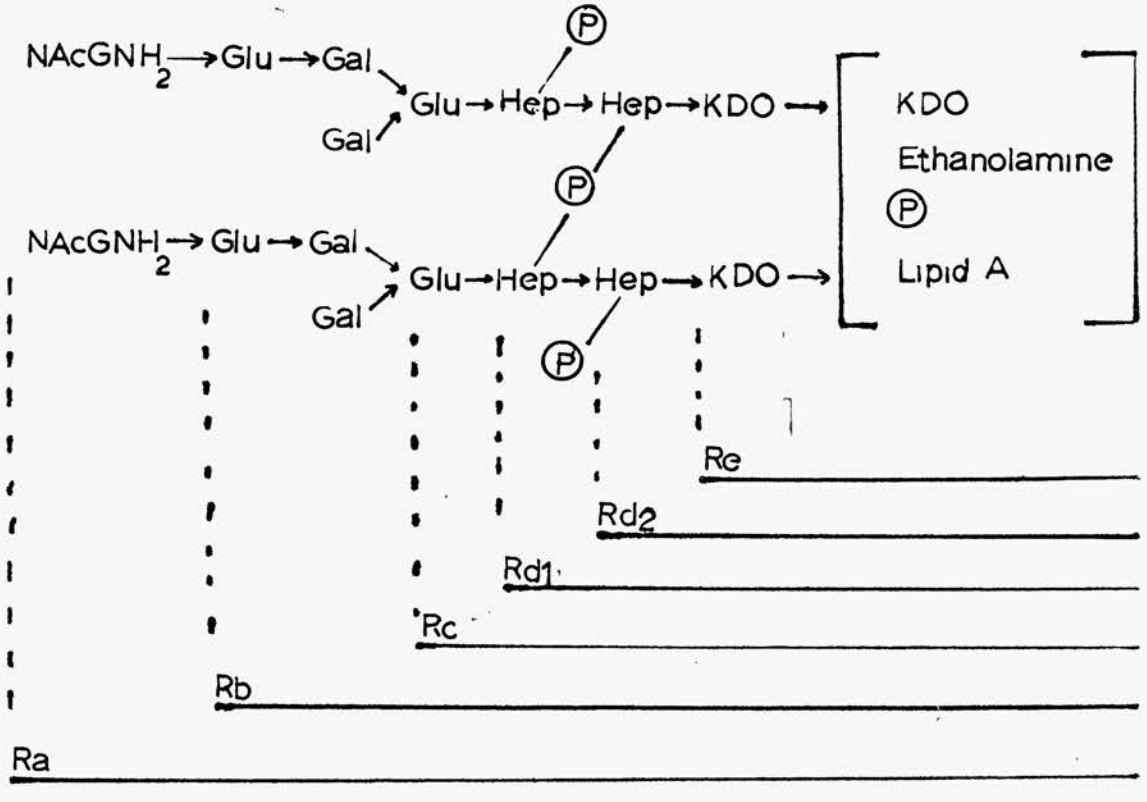


FIGURE 16 DIAGRAMMATIC REPRESENTATION OF *Salmonella* R-MUTANTS



proposed that the basic minimum structure of the LPS was a polyheptose KDO backbone. It is now known that mutants devoid of even heptose exist (Lüderitz et al., 1966; Stocker, Wilkinson and Mäkelä, 1966) with a LPS containing KDO as the only sugar component, these mutants being as yet the simplest discovered.

From partial acid hydrolysates of the RII mutant a number of oligosaccharides have been characterised (Sutherland, Lüderitz and Westphal, 1965) and a structural unit proposed for the RII LPS (Figure 15), the unit believed to be attached to the polyheptose-KDO backbone proposed by Osborn (1963). On the basis of these results Sutherland et al. proposed that Salmonella spp. contain a common core structure similar to that suggested for RII mutants, and that an outer core region carrying the O antigenicity is attached, possibly to the $NAcGHN_2$ residue. RI mutants lack the $NAcGNH_2$ residue, and therefore the necessary part of attachment for the O specific chains, and the RII mutants are believed to have a defect in O specific chain synthesis. Further evidence for this general theory was the observation that R specificity could be shown to be present in smooth serotypes when the LPS was partly degraded by acid hydrolysis, suggesting that the O antigenic determinants were being stripped off, revealing the underlying core structure (Lüderitz, Beckmann and Westphal, 1964).

The innermost region of the core is still not known in any detail, particularly as to how the heptose phosphate KDO region is linked to lipid A, or another compound, ethanolamine

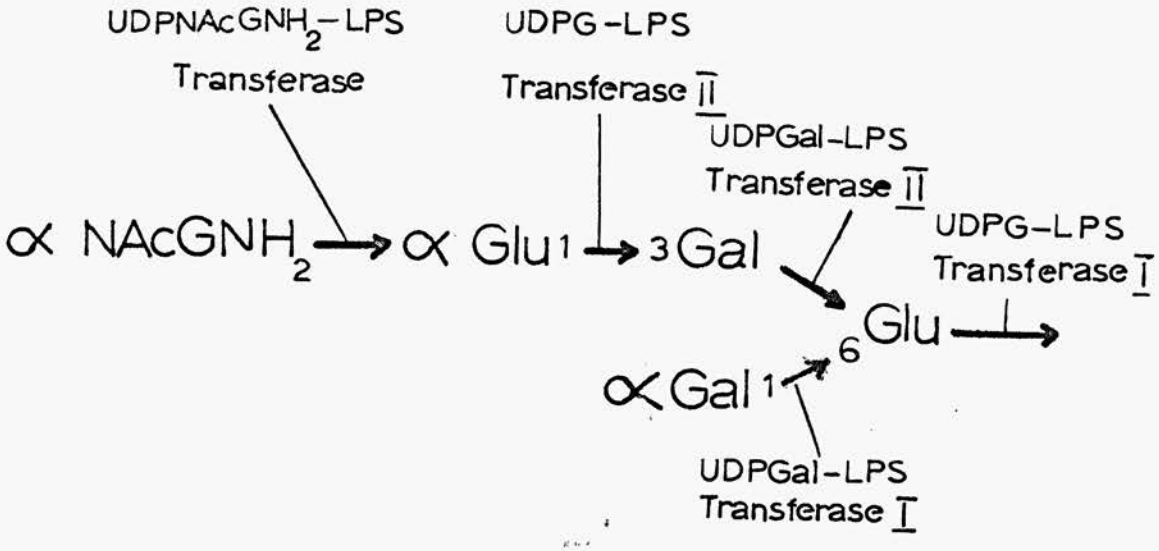
phosphate, which has been isolated recently and believed to be an integral part of the innermost region (Grollman and Osborn, 1964). Various oligosaccharides have been isolated (Cherniak and Osborn, 1966; Risse et al., 1967; Dröge, Lüderitz and Westphal, 1967) which suggest that the core unit of Sutherland, Lüderitz and Westphal (1965) is linked through the terminal glucose by a 1→3 linkage to a heptosyl 1→3 heptosyl 1→7 or 1→8 KDO unit, but qualitative data indicate more KDO in the LPS, and its site of attachment is unknown (Lüderitz, Jann and Wheat, 1968). The heptose found in most LPS was first identified by Weidel (1955) and has been identified as L-glycero-D-mannoheptose (Osborn et al., 1964) although other heptoses are known such as D-glycero-D-galactoheptose in Chomobacterium violaceum (Davies, 1960) and D-glycero-D-mannoheptose in Proteus murabilis (Bagdian et al., 1966). Phosphodiester bridges are believed to link adjacent heptose-KDO units (Osborn, 1963; Lüderitz, Jann and Wheat, 1968) giving a chain of polyheptose KDO units, forming a backbone for the attachment of other core sugars. There is some evidence that KDO serves as the linking group between polysaccharide and lipid, since KDO appears as reducing groups on liberation of lipid A (Fraenkel et al., 1963) and KDO is incorporated from CMPKDO into a partially degraded lipid acceptor, possibly through a hydroxyl group of glucosamine or β-hydroxy myristic acid (Mayer, Edstrom and Heath, 1965) but no one has yet isolated fragments showing the linkage, nor for that matter indicating the attachment of ethanolamine phosphate.

Numerous chemically distinct R forms have now been recognised and investigated, particularly by Luderitz and his group, and a diagrammatic representation of the core polysaccharide is shown in Figure 16 indicating the structures of the various R forms. The most minimal LPS is produced by Re mutants (Lüderitz et al., 1966; Stocker, Wilkinson and Mäkelä, 1966) containing only lipid A, ethanolamine phosphate, and KDO. Rd mutants have been obtained, Rd_1 types being unable to make UDPG similar to the original mutants described by Fukasawa, Jokura and Kurahashi (1962) (Sundarajan, Rapin and Kalckar, 1962; Fraenkel et al., 1963), and another type lately described in which the ability to make UDPG is not impaired, but the organism lacks the necessary transferase to catalyse the transfer of glucose to the heptose backbone (Risse et al., 1967). Rd_2 mutants have only very recently been described, having only half the heptose content of the Rd_1 types, presumably lacking a heptose transferase (Risse et al., 1967). Rc mutants include the strain of Osborn (1963), being unable to make UDPGal, and a mutant described as lacking the necessary transferase for the incorporation of galactose is also of this type, although it has been shown that such mutants have galactose into the LPS in the $1 \rightarrow 6$ linkages attached to the innermost glucose, so clearly there are two transferases catalysing the incorporation of galactose, as might be expected from the structure indicated (Risse, Luderitz and Westphal, 1967; Osborn, 1968). Rb mutants are equivalent to the RI serological type, lacking

$NAcGNH_2$, and Ra mutants are equivalent to the RII type, being unable to synthesise or attach the O antigenic side chains. The nature of the mutation involved in RI strain has not been reported, presumably it is a defect in $NAcGNH_2$ synthesis, or in its transfer to the distal glucose of the core. In most examples the defect producing RII mutants has also not been reported or studied. In one case the wild type organism produces a rhamnose containing O antigenic side chains, and the RII derivative was shown to have a defect in TDP rhamnose synthetase (Nikaido et al., 1964) and a strain of S. typhimurium studied by Osborn et al. (1964) gave an RII mutant unable to synthesise GDP mannose for incorporation into O antigenic chains. The only other report is of a strain of S. minnesota designated R60, presumably an RII type, which lacks the enzyme $UDPNAcGNH_2$-4-Epimerase which is required for the synthesis of $NAcGalNH_2$, one of the type specific sugars (Lüderitz et al., 1964).

The chemically defined structure for the core polysaccharide has been confirmed by a brilliant series of investigations by several groups of workers on the biosynthesis of the structure, which took place in conjunction with the chemical investigations. Experimental approach to the biosynthesis of these complex molecules has been greatly facilitated by the availability of the mutant organisms already discovered, in which an incomplete LPS is synthesised. Application of such techniques was made possible by the observations of Nikaido and his co-workers (Nikaido, 1961; Fukasawa and Nikaido, 1961)

FIGURE 17 DESIGNATION OF SUGAR NUCLEOTIDE – LPS TRANSFERASES IN LPS CORE SYNTHESIS



concerning certain galactose-negative mutants of Salmonella, which were unable to synthesise UDPGal because of a defect in UDPG-4-Epimerase. These mutants, which would now be categorised as Rc, formed an incomplete LPS containing only glucose, heptose and KDO, unless grown on galactose media, when galactose was quickly transferred to the LPS, the composition returning to normal. Furthermore, for the first time it was demonstrated (Nikaido, 1962a; 1962b) that cell-free extracts of the mutant were able to carry out the incorporation of galactose from UDPGal into the galactose deficient material of the LPS. Similar results were reported by Osborn et al. (1962) with an analagous mutant, and the galactose was shown to be linked 1 → 3 with the glucose already present in the LPS (Rosen, Osborn and Horecker, 1964). With the same organism, sequential transfer of galactose, glucose and N-acetyl glucosamine from the corresponding UDP derivatives has been demonstrated (Osborn and D'Ari, 1964) strongly supporting the core structure derived by chemical means. The transfer of the first glucose residue to the heptose backbone has also been demonstrated using two different mutants, both unable to make UDPG (Rd_1). One strain is deficient in phosphoglucose isomerase (Fraenkel et al., 1963) and the other in UDPG pyrophosphorylase (Fukasawa, Jokura and Kurahashi, 1962). Cell-free preparations of such organisms catalyse transfer of glucose from UDPG into the incomplete LPS (Rothfield, Osborn and Horecker, 1964). The enzymes have been designated (Figure 17) as glucosyl transferase I, galactosyl

transferase II, glucosyl transferase II, and $NAcGNH_2$ transferase, with the enzyme which adds galactose in a 1 → 6 linkage to the innermost glucose, known as galactosyl transferase I (Osborn and D'Ari, 1964; Osborn, 1968). All the enzymes, with the exception of galactosyl transferase I, have been demonstrated in cell-free systems, and two of these enzymes, glucosyl transferase I and galactosyl transferase II, have been obtained in soluble form, making it possible to examine their specificities (Osborn et al., 1964; Osborn and Horecker, 1964). Attempts to purify the LPS acceptor for these enzyme systems by the usual methods such as phenol extraction (Westphal, Lüderitz and Bister, 1952) and demonstrate activity failed, suggesting an additional factor was involved. This factor was subsequently identified as phospholipid (Rothfield and Horecker, 1964; Rothfield and Takeshita, 1965), the active acceptor molecule being characterised as a LPS-phospholipid complex, which could be generated from the individual components by mixing at 60^{o} and cooling slowly. It was suggested that a lipid B derivative was involved in the cell, and that an enzyme-phospholipid-LPS complex was bound to the apposite sugar nucleotide (Rothfield and Takeshita, 1966). Horecker (1966) has suggested that selective adsorption of specific transferases may occur, each transferase being released on completion of the reaction, an active site being exposed, ready for the next transferase-sugar nucleotide reaction to take place.

The O-antigenic side chain structures determined so far

FIGURE 18 O-ANTIGENIC SIDE-CHAIN OF S. typhimurium

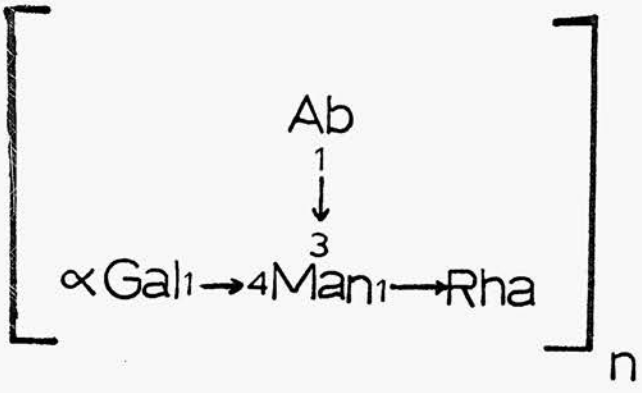
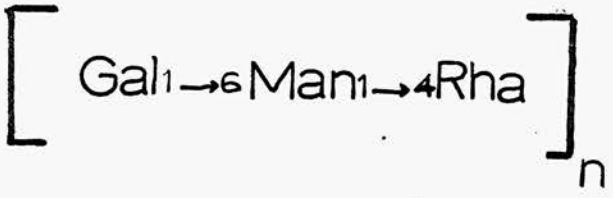


FIGURE 19 O-ANTIGENIC SIDE-CHAIN OF S. anatum



are legion (Lüderitz, Jann and Wheat, 1968), and initially it was thought that the biosynthesis of the units proceeded in the same way as the core, by the stepwise addition of the specific sugars. Initial studies on the biosynthesis of the O antigen in the TDP rhamnose deficient strain of S. typhimurium appeared to bear this out. The O-antigenic side chain is illustrated in Figure 18 (Robbins and Uchida, 1962; Tinelli and Staub, 1959; 1960) and Nikaido (Nikaido, 1965; Nikaido and Nikaido, 1965) was able to demonstrate the incorporation of galactose and rhamnose from UDPGal and dTDP rhamnose into LPS, followed apparently sequentially by mannose from GDPM and abequose from CDPAb. However, other evidence seemed to point against sequential transfer of sugars, in particular the apparently simultaneous deletion of all O antigenic sugars, following a single mutation such as the loss of TDP rhamnose synthetase. The argument might be that rhamnose was of particular importance as the first sugar in the chain, hence the reason that none of the other sugars were incorporated, but despite the very large number of rough mutants isolated, lacking the entire O complex, a mutant synthesising an incomplete O-antigen has not been isolated in contrast to the many various incomplete core mutants. Another important observation was made by Beckman and his co-workers (Beckman, Subbaiah and Stocker, 1964) that RI (Rb) mutants contain a water soluble hapten with O specificity, strongly suggesting that sugars may not be transferred sequentially to the outermost region of the core, but

polymerised separately by some other mechanism, perhaps as nucleoside diphosphate oligosaccharides (Sutherland, Luderitz and Westphal, 1965).

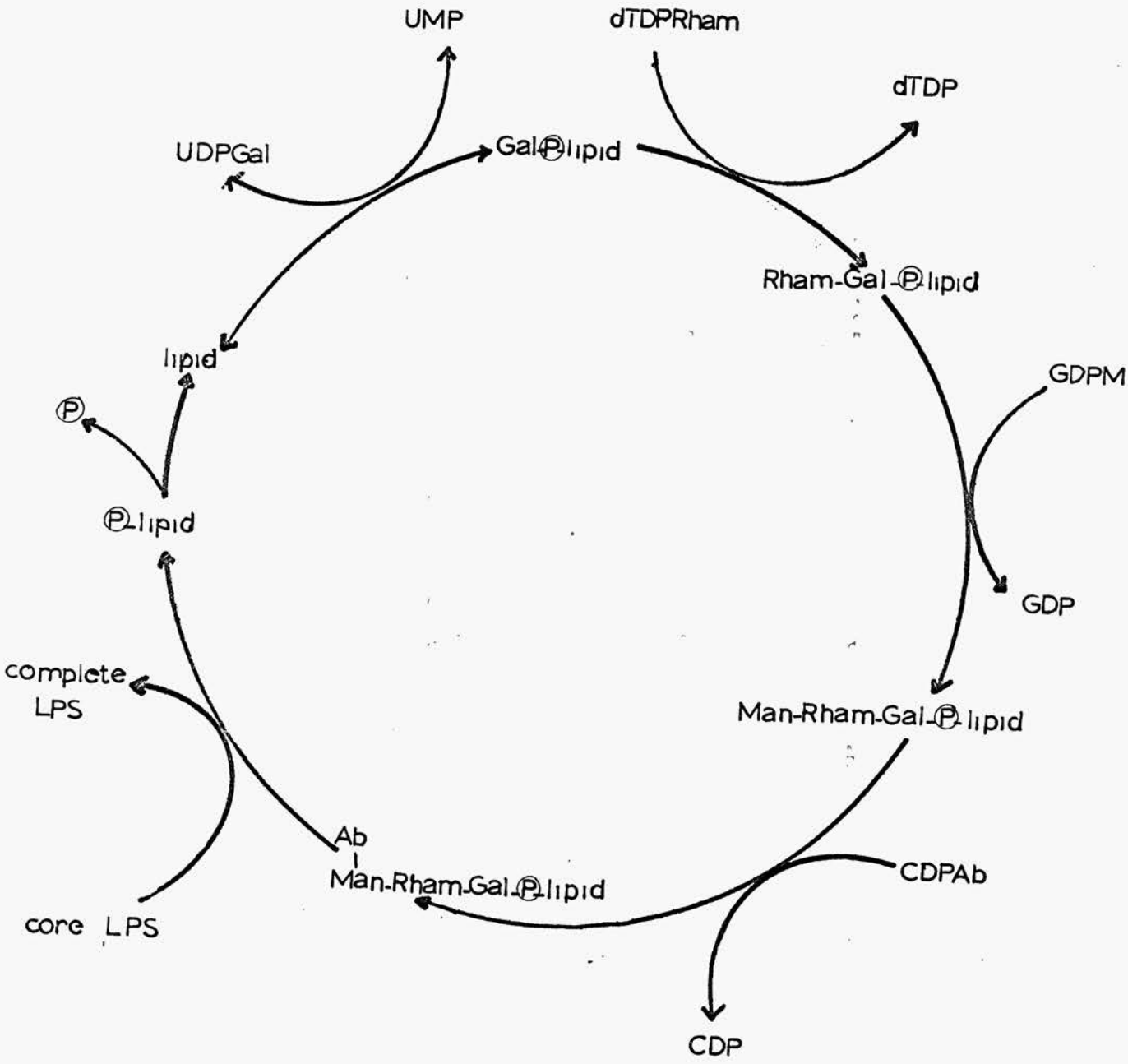
Further biosynthetic experimental evidence was to clarify these points, several groups working almost simultaneously on this problem. Zeleznick et al. (1965), working with a strain of S. typhimurium unable to synthesise GDPM, reported that incorporation of mannose, rhamnose and galactose was obtained from the apposite nucleotide derivatives, and they obtained galactosyl-mannosyl-rhamnose oligosaccharides from the product. Abequose incorporation was not tried at this point, perhaps due to unavailability of CDPAb, and it was assumed that since abequose is on a branch chain, it was unnecessary for the synthesis of a product containing the other three sugars. In contrast to the results of Nikaido and his group, Zeleznick et al. reported that whereas some small incorporation of galactose and rhamnose occurred in the absence of GDPM, for maximal incorporation of any sugar, the other two had to be present as nucleotide derivatives.

At almost the same time Robbins and his group were investigating the biosynthesis of the S. anatum O-antigen using a wild type strain. The structure of the antigen is shown in Figure 19, and it had been observed that incorporation of any of the component sugars, galactose, mannose and rhamnose, from sugar nucleotides, required the presence of the others (Robbins, Wright and Bellows, 1964) similar to the results of Zeleznick

et al. (1965). Later work with S. newington which is the same chemotype as S. anatum showed this interdependence of incorporation again, and this time the interdependence was investigated. On omission of GDPM from the reaction mixture, no incorporation of the other two sugars was observed into LPS, but extensive incorporation was observed into a dialysable fraction which was tentatively identified as rhamnosylgalactosylphosphate acceptor (Wright, Dankert and Robbins, 1965). On addition of GDPM, polymerisation to non-dialysable material attached to LPS occurred. This intermediate acceptor was characterised as a lipid of molecular weight about 1000, containing a phosphodiester bridge, linking it to the disaccharide (Dankert et al., 1966).

Further investigation of the S. typhimurium system was to clarify the mechanism. This time the group (Weiner et al., 1965) used an Rc mutant with a deficient core polysaccharide, in order to prevent transfer of any O-antigenic material synthesised, into the core. It was shown that on addition of UDPGal to a particulate cell-free system, UMP was released and a galactosylphosphate lipid was formed, to which could be added rhamnose from TDPRh to give a disaccharide phosphate lipid, and that further addition of mannose from GDPM gave a trisaccharide which was quickly polymerised to give material of O-antigenic specificity. This material was different from native O-antigen since it lacked abequose, but it was again concluded that since abequose occurs as a branch, it was not necessary for

FIGURE 20 ROLE OF LIPID-LINKED INTERMEDIATES IN *S. typhimurium* O-ANTIGEN BIOSYNTHESIS



polymerisation. Later work (Weiner et al., 1966) suggested that abequose could be incorporated from CDPAb to give a transient tetrasaccharide which was polymerised.

The lipid acceptor has been further characterised (Wright et al., 1967) as a C_{55} polyprenoid alcohol, linked to the sugars by pyrophosphate, striking similarities between this compound and the compound described by Dietrich, Colucci and Strominger (1967) participating in murein biosynthesis being evident. In the light of this evidence for O-specific oligosaccharides, the water soluble hapten exhibiting O-specificity found in RI (Rb) mutants takes on new significance, and work is in progress to determine if this water soluble hapten is related to the lipid-linked intermediates, Weiner et al. (1966) having pointed out that in S. typhimurium both the hapten obtained from RI (Rb) derivatives and the lipid-linked intermediates in O-antigen biosynthesis have galactose at the reducing end. The O-antigenic biosynthetic system is outlined in Figure 20, emphasizing the parallel between LPS biosynthesis and murein biosynthesis.

Nikaido has re-examined and repeated his studies on O-antigenic chain biosynthesis in the TDP Rhamnose deficient strain of S. typhimurium, again showing incorporation of galactose, rhamnose and mannose into LPS, followed by abequose, but has been unable to detect any lipid intermediates (Nikaido, Naide and Makela, 1966). In this system incorporation of galactose and rhamnose alone, was observed again, and Nikaido

et al. attribute this to the method of preparation of the enzyme system. It was supposed that the specificity of the oligosaccharide transferase is altered by their method of preparing a cell-free extract, and that, as a result, an incomplete galactosyl-rhamnose chain is synthesised, in the same way that both Nikaido (Nikaido, 1965; Nikaido and Nikaido, 1965) and the Horecker group (Zelezniek et al., 1965; Weiner et al., 1965) have observed synthesis of a galactosyl-rhamnosyl-mannose chain, in the absence of abequose. The point was also made that since these mutants contain a complete core structure, very rapid incorporation into LPS may have taken place, making lipid intermediates difficult to detect.

Work has also been in progress on the biosynthesis of LPS in an E. coli strain deficient in UDPG-4-Epimerase (Edstrom and Heath, 1964), and incorporation of galactose, glucose, $NAcGNH_2$ and colitose from the apposite nucleotide donors has been observed in cell-free systems (Edstrom and Heath, 1964; Mayer et al., 1966; Edstrom and Heath, 1967) but it is not clear if synthesis of the core region, or the O-antigenic region, is occurring, since in this organism the core and O-antigenic region are very similar containing glucose, galactose and $NAcGNH_2$ in common (Edstrom and Heath, 1967). Colitosyl oligosaccharides have been obtained from the reaction product, and there is some evidence for a lipid bound colitose intermediate (Mayer et al., 1966), possibly containing glucose, colitose and $NAcGNH_2$. However, the system remains to be clarified, and the

structure of the LPS to be worked out fully.

In any general consideration of O-antigenic side chain biosynthesis, several points require clarification. In the S. anatum and S. newington system, presumably addition of oligosaccharide units occurs to incomplete O-antigen chains already there, since these systems were derived from smooth, wild-type organisms, and it is not clear if they are added individually, or if sections are polymerised to an optimum size and then added. In the S. typhimurium system described by the Horecker group, attachment of chains to the core LPS cannot occur, and polymerisation of the units occurs to give high molecular weight material. This material has not yet been shown to become attached to core LPS in cell-free systems, and its relationship to O-antigen biosynthesis in vivo remains to be established. It has been pointed out that, if sequential addition occurs (Naide et al., 1965; Horecker, 1966), a separate enzyme may be required to add the first oligosaccharide unit to the core since $NAcGNH_2$ is presumably the acceptor, and all subsequent transfers will be to the terminal sugar of the preceding unit. Semi-rough mutants have been isolated (Naide et al., 1965) which apparently have only one oligosaccharide unit attached to the core, consistent with this view of sequential addition. Conversely, there is the evidence of the water soluble hapten accumulated by RI (Rb) mutants which is probably polymerised material, since the semi-rough mutants, while presumably able to produce the necessary oligosaccharide

FIGURE 21 DIAGRAMMATIC REPRESENTATION OF TWO POSSIBLE MECHANISMS OF POLMER CHAIN ELONGATION

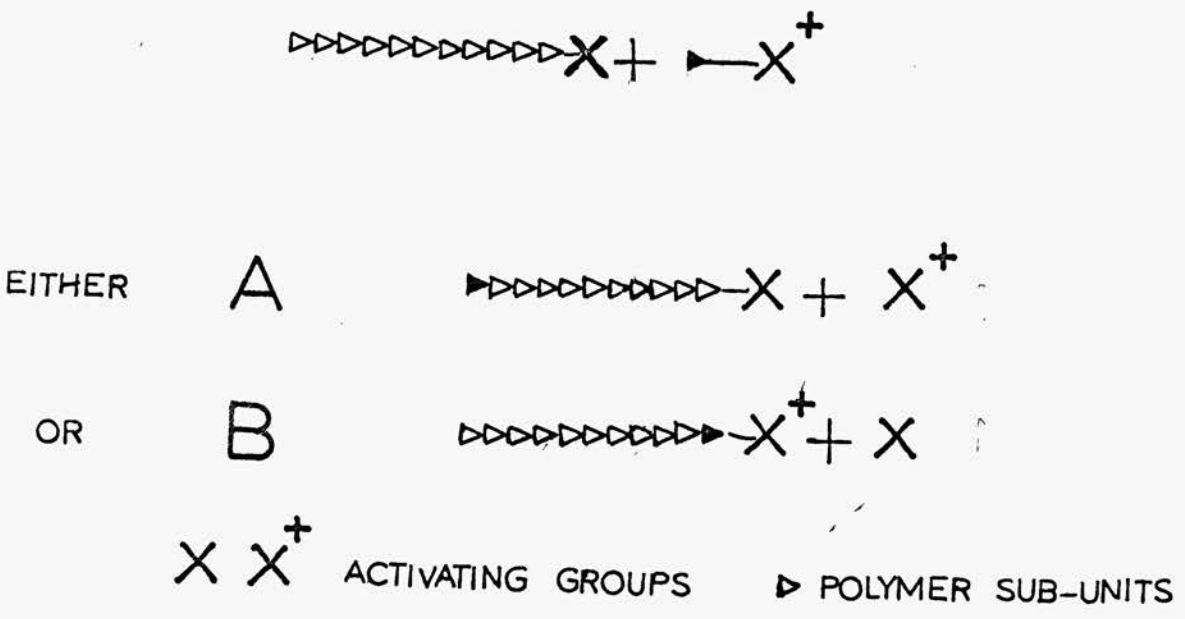
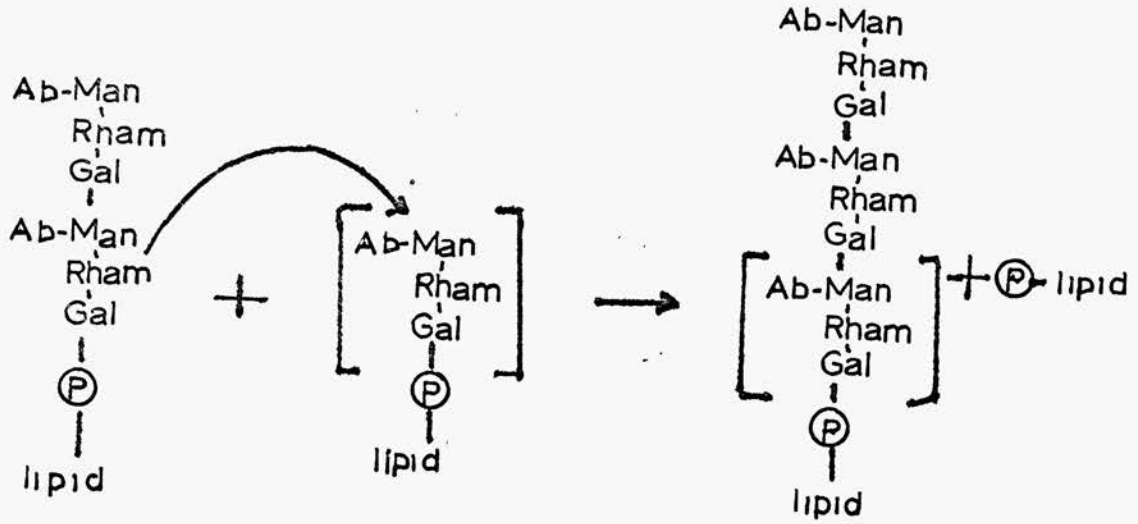


FIGURE 22 MECHANISM OF CHAIN ELONGATION IN. S typhimurium O-ANTIGEN



intermediates, do not produce a hapten. This seems to point to the fact that the water soluble hapten is not simply an accumulation of lipid-linked intermediates, or their break down products, a strong piece of evidence for polymerisation, then addition to the core.

Recently the mechanism of chain elongation in O-antigen biosynthesis has been further discussed (Bray and Robbins, 1967; Robbins et al., 1967). In the case of many homopolysaccharides, monosaccharide units, activated at the reducing end, usually by means of a nucleoside diphosphate, are added in step-wise fashion to the non-reducing end of the polymer, and this mechanism has been generally taken to be universal in the biosynthesis of polysaccharides (Ginsburg, 1964; Neufeld and Hassid, 1963). In the case of heteropolymers, synthesised by the polymerisation of preformed oligosaccharides, Robbins et al. point out that there is no reason to believe that addition occurs at the non-reducing end, and on the basis of pulse labelling experiments, conclude that in O-antigen biosynthesis, activated chains are added to the non-reducing end of sub-units. The basis of this argument is that there are two possibilities for the building up of a chain (Figure 21) and to determine the direction of growth, Robbins et al. determined which end of the chain became labelled with ^{14}C in a series of experiments. The evidence suggested that addition occurred to the activated reducing end of the chain, the second possibility in Figure 21, illustrated for the O-antigen biosynthesis in S. typhimurium

in Figure 22. It has been suggested that, in the light of this result, many polysaccharide systems require to be examined, to determine if this occurs as a general rule, since, apart from a few systems such as starch, glycogen, xylodextrins and LPS core where unequivocal evidence exists for addition to the non-reducing end, many systems have been assumed to be similar without any real evidence.

BACTERIAL GLYCOGEN

An intracellular polyglucose, similar to typical animal glycogen, is present in a wide variety of Gram-positive and Gram-negative bacteria, sometimes in relatively high concentrations (Sigal, Cattaneo and Segel, 1964; Gibbons, 1964; Ghosh and Preiss, 1965). Preiss and his group, in a comprehensive series of experiments, have shown that whereas UDPG is the donor in animal glycogen synthesis, in bacteria the donor is ADPG, in a reaction essentially similar to that involved in the synthesis of animal glycogen (Shen et al., 1964; Greenberg and Preiss, 1964; Shen and Preiss, 1965; Greenberg and Preiss, 1965). Several genera have been investigated, and ADPG-glycogen glucosyl transferase is of wide occurrence (Ghosh and Preiss, 1965). Previously, in a strain of E. coli unable to synthesise UDPG, it had been shown that glycogen accumulated, indicating that a precursor other than UDPG was involved (Sigal et al., 1964). A number of 1 4 glucans serve as primers for the reaction (Greenberg and Preiss, 1965), ranging from maltodextrine to rabbit liver glycogen, and a branching enzyme of the type associated with the synthesis of animal glycogen and phytoglycogen (Lavintman, 1966), has been isolated from E. coli (Sigal et al., 1965; Zevenhuizen, 1964). Shen and Preiss (1965) have studied the glycogen synthetase of various organisms, and ADPG pyrophosphorylase, and found that in contrast to mammalian systems, glycogen synthetase does not appear to be

activated by certain phosphorylated intermediates of metabolism such as G-6-Ⓟ but that the pyrophosphorylase is strongly activated by several compounds, indicating that control of synthesis is probably at the pyrophosphorylase level.

EXTRACELLULAR POLYSACCHARIDES

Both the Gram-positive and the Gram-negative bacteria may produce copious amounts of polysaccharide material, either as a capsule surrounding the cell, or as a slime found dissolved in the culture medium. In pathogenic bacteria, there is a well established correlation between encapsulation and virulence, apparently due to encapsulation rendering the bacteria less susceptible to phagocytosis (Smith, 1927; Ewing, 1956; Leidy et al., 1960; Bhatnagar, Speechly and Singh, 1938; Burrows and Bacon, 1956) and many bacteria are found to be encapsulated when freshly isolated from biological material. Capsules may also protect bacteria against attack by bacteriophage, since often the receptor site for the phage is on the cell wall or envelope, but capsular material may itself be the substrate of phage-induced enzymes which remove the capsular material, uncovering the cell surface which is usually the phage receptor (Humphries, 1948; Adams and Park, 1956; Edlinger and Vieux-change, 1953; Taylor, 1963; Bernard, 1965a, 1965b; Sutherland and Wilkinson, 1965).

The polysaccharide material is usually negatively charged, due to the presence of phosphate, hexuronic acid, aminohexuronic acid, or sialic acid, and it has been known for a considerable length of time that such extracellular polysaccharides are not essential structural features of the cell, and that their production is subject to environmental conditions. The

anatomical and functional differentiation of capsular polysaccharide from the cell wall or cell envelope was established by some early studies on Pneumococcus, in which the capsular polysaccharide was stripped from the cells of a particular strain, by means of a specific enzyme, without impairing the viability of the cells (Avery and Dubos, 1931). Similar results have been reported for Klebsiella (Adams and Park, 1956) and Bacillus (Torii, 1955). The environmental conditions have been shown to be of extreme importance in determining polysaccharide production, and Duguid and Wilkinson (1953; 1954) have shown that polysaccharide production was markedly enhanced under conditions of carbon excess, and limitation of nitrogen or phosphorus, as evidenced by the diameter of the capsule of a strain of Klebsiella. Later experiments (Wilkinson, Duguid and Edmunds, 1954) showed that lowering the temperature of growth to 15-20°C also markedly enhanced polysaccharide production.

Well developed capsules can be visualised using light microscopy, and a great number of staining methods have been developed for their demonstration (Duguid, 1951). Many encapsulated cells produce slime which is not attached to the cell surface, and there is no definite distinction between slime and capsular material, some types of bacteria producing well-defined capsules, others copious amounts of slime, without any capsular structure being visible (Duguid and Wilkinson, 1953; 1954; Wilkinson, 1958). In some cases a capsule is

present which cannot be observed by light microscopy, and yet may be detectable by other means. In these cases the term "microcapsule", introduced by Wilkinson (1958), is applied. In such cases there is the possibility, particularly if the detection method is serological, that the material classified as a "microcapsule" may be in reality an integral part of the cell wall or cell envelope. The O-antigens of the Enterobacteriaceae, and the M-antigens of the haemolytic Streptococci have been placed in this group, and clearly the term "microcapsule" has no real significance in these cases (Salton, 1964). The term can only have any real use in those cases where there is a definite structure, separate from the cell wall or cell envelope, and this never assumes the dimensions of a true capsule, such as the V_i antigen of S. typhi.

The literature abounds with reports of the isolation of mucoid strains of bacteria, and reports on the capsule or slime composition. The extracellular material can range from the simple homopolysaccharide type of polymer, to the complex heteropolysaccharides of the Klebsiella, Escherichia and Pneumococcus groups. An organism may produce more than one type of extracellular polysaccharide as in some Klebsiella types (Eriksen, 1966) and Serratia marcescens (Adams and Young, 1965; Adams and Young, 1966). In S. marcescens, three different polysaccharides are found, each containing fatty acids and protein as integral parts of the molecule, and this is interesting in view of the purification techniques used by

ABBREVIATIONS USED IN TABLE 4

glu: D-glucose

glu A: D-glucuronic acid

4-methoxyl glu A: 4 methoxy-D-glucuronic acid

gal: D-galactose

fuc NH_2: L-fucosamine

man A: D-mannuronic acid

GNH_2: D-glucosamine

$GalNH_2$: D-galactosamine

$NAcGNH_2$: N-acetyl-D-glucosamine

$NAcGalNH_2$: N-acetyl-D-galactosamine

$NAcGalNH_2A$: N-acetyl-D-galactosamineuronic acid

MNH_2: D-mannosamine

rham: L-rhamnose

man: D-mannose

$NAcfucNH_2$: N-acetyl-L-fucosamine

gul A: L-guluronic acid

NANA: N-acetyl neuraminic acid

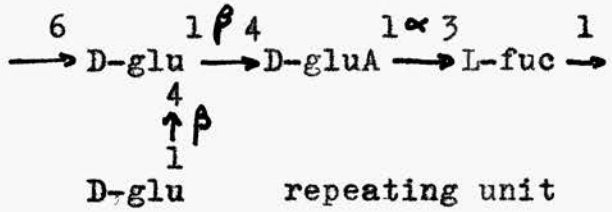
P: phosphate

fuc: L-fucose

gal A: D-galacturonic acid

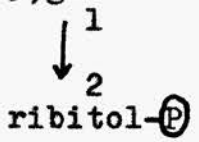
hex NH_2: hexosamine

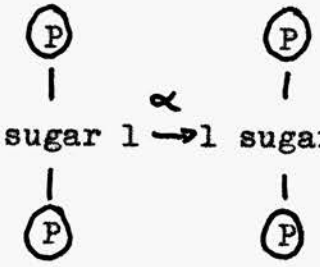
TABLE 4: EXOPOLYSACCHARIDES OF BACTERIA

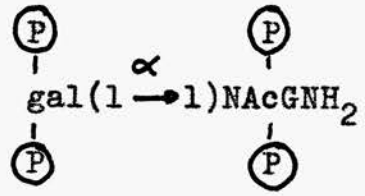
Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Klebsiella	1	glu A, gal, glu, fuc, pyruvic acid	1,2,3,4, 5,6,20	-	
	2	glu A, glu, rham, pyruvic acid	1,2,3,4, 5,6,7,20	-	
	3	gal A, man, gal, pyruvic acid	1,2,3,4, 5,8,9,10, 11,12,20	Gal A → man gal → 4 man linkages	10
	4	gal, glu, man, pyruvic acid	1,12,20	-	
	5	gal, glu, man, pyruvic acid	5,20	-	
	8	gal, glu	13	-	
	26	gal, glu, man	13	-	
	29	gal, man	13	-	
	54	glu A, fuc, glu	14		18,19
	57	gal, man	13	-	
	64	glu A, glu, man, rham	15,16	-	
	11	gal, glu, man	17	-	
	21	gal, man	17	-	

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
E. coli	K34	gal, glu, man, hex NH_2	32	-	
	K42	gal A, gal, fuc, O acetyl	30,31,33, 34	gal(1→3)gal A(1→2)fuc repeating unit	34
	K85	glu A, man, $NAcGNH_2$, rham	30,31,33, 35	glu A(1→2)man(1→3)man(1→3) with rham on man(1→2), rham and $NAcGNH_2$ on man(1→3)	35
	K 1	NANA	90,20,38	-	
	?	NANA, GNH_2, gal, glu	20	-	
	?	NANA, gal, gal NH_2	37	-	
	K 7	MNH_2, GNH_2, glu, gal	20	-	
Pneumococcus	I	gal, fuc, $NAcGNH_2$, gal A, O acetyl	65	-	
	II	glu A, glu, rham	66,67,68	-	
	III	glu, glu A	69	glu A β1→4 glu β1→3 glu β1→4 glu A repeating unit	70
	VIII	gal, glu, glu A	71	glu A β1→4 glu β1→4 glu β1→4 gal repeating unit	71

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Klebsiella NCTC 418		glu A, glu, man, man A	21	glu A 1→4 man Man A 1→4 Glu linkages	21
Escherichia coli	K27	glu A, gal, glu, man, fuc	30,31	-	
	K30	glu A, gal, man, fuc	30,31	man(1→2)glu A(1→2)gal	93
	K87	glu A, GNH_2, $FucHN_2$, gal, glu, rham	30,31	-	
	K 4	gal A, $galNH_2$, gal, glu	30,31	-	
	K 8	glu A, $galNH_2$, GNH_2, gal, man	30,31	-	
	K34	gal, glu, man	32	-	
	K17	glu A, GNH_2, gal, glu	30,31	-	
	?	GNH_2, gal, rham, glycerol, P	20	-	
	?	NANA	20	NANA(1→8)NANA linkages	20
	?	man, glu, gal, glu A	36	glu → man → glu → man ↑ glu ↑ man ↑ glu A	36
	K28	gal, glu, man, hex NH_2, fuc	32	-	

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Pneumococcus	XVB	gal, glu, GNH_2, ribitol-P, glycerol, O-acetyl	84	-	
	XVI	gal, glu, GNH_2, rham, glycerol, P, acetyl	84	-	
	XIX	glu, gal, GNH_2, ribitol-P, pentose, O-acetyl	84	-	
	XX	glu, gal, GNH_2, ribitol-P, O-acetyl	84	-	
	XXI	glu, gal, GNH_2, rham, P, O-acetyl	84	-	
	XXVII	glu, gal, GNH_2, uronic acid, rhamnose, P, O-acetyl	84	-	
	XXX	glu, gal, $galNH_2$, ribitol-P, O-acetyl	84	-	
	XVIII	glu, gal, rham, glycerol, P, ribitol	82,83	gal(1→4)glu(1→6)glu(1→3) rham(1→4)glu	83
	XXXIV	galactofuranose, glu, ribitol-P	84,85,86	gal furan(1→3)glu(1→2)gal furan(1→3)gal 	84,85,86
	IV	gal, amino sugar, O-acetyl	87	-	
	X	gal, GNH_2, ribitol-P, O-acetyl	84	-	

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Pneumococcus	XA	galactofuranose, $galNH_2$, ribitol-P	84,88	-	
	XI	gal, glu, GNH_2, $galNH_2$, ribitol-P, O-acetyl	84	-	
	XIA	glu, gal, glycerol, P, O-acetyl	84	-	
	XII	glu, gal, amino sugar, O-acetyl	84	-	
	XIII	glu, gal, GNH_2, ribitol-P, O-acetyl	84	-	
Haemophilus influenzae	a	glu, P	39		40,41,43
	b	ribose P	39,40,41		
	c	hexose P	39		
	f	$galNH_2$ P	41		
	d	$NAcGNH_2$, $NAcGalNH_2A$	42		
	e	hexose (L glucose?) GNH_2	41		
Salmonella typhi and a few other members of the Entero-bacteriaceae	V_i	$NAcGalNH_2A$	22,23	1 - 4 linkages	25
		O-acetyl	24		
Vibrio ?	-	hexosamine, O-acetyl	29	-	

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Haemophilus suis	-	$NAcGNH_2$, gal, P	42		42,44
Neisseria meningitidis	-	NANA, GNH_2	45,46	-	
Pseudomonas aeruginosa	-	fuc, glu, gal, GNH_2, $galNH_2$, hexosamine	47,48	-	
	-	fuc, glu, gal, GNH_2, $galNH_2$, NANA	47,48	-	
	-	man A, gul A, O-acetyl	50,51,91	-	
	-	glu A, gal, man, rham	53,54	-	
Leuconostoc mesenteroides and many other bacteria grown on sucrose		glucose	108	α 1 → 6 linkages	108
Zymomonas mobilis, Aerobacter hevanicum, and other spp. grown on sucrose		fructose	98,108	β 2 → 6 linkages	98,108

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Sphaerotilus natans		glu, gal, glu A, fucose	106	-	
Citrobacter freundii		NANA, $NAcGNH_2$, $NAcfucNH_2$	100	-	
Rhizobium meliloti		glu, gal, glu A	89	-	
R. leguminosarium		glu, glu A	53,54,103	-	
R. trifolii		glu, glu A, 4 methoxy glu A	53,54	-	
R. phaseoli		glu, glu A, 4 methoxy glu A	53,54,103	-	
R. lupinii		man, glu, rham, 4 methoxy glu A	103	-	
R. japonicum		glu, rham	103, 62	-	
Serratia marcescens		(a) glu A, glu, man	58		
		(b) rham, glu	105		
		(c) D-glycero-D-mannoheptose, L-glycero-D-mannoheptose		-	
		(d) rham, glu, heptose			
Pasteurella multocida		fructose, glu, man, GNH_2	59	-	

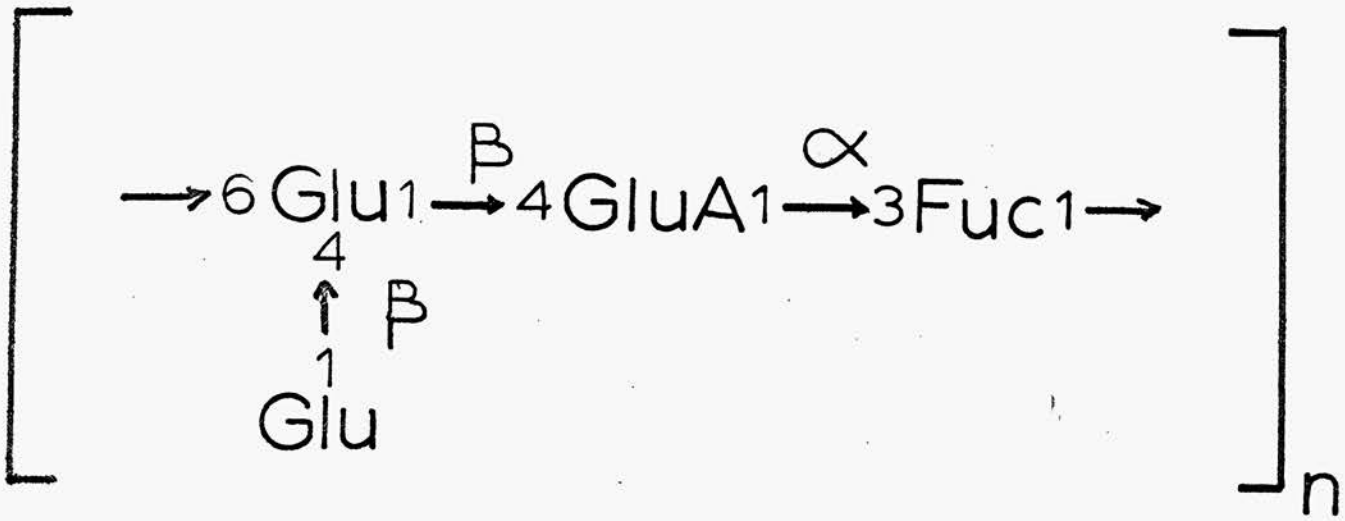
Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Azotobacter vinelandii		gal A, man A, rham, O-acetyl, 2 keto 3 deoxygalactonic acid	55 56	-	
A. chroo-coccum		glu	64	-	
A. agilis		gal, rham, 2 keto 3 deoxy-galactonic acid	57	-	
Arthrobacter viscosus		gal, glu, man A, O-acetyl	101	-	
A. globi-formis		glu A, glu	102	-	
Strepto-coccus bovis		glucose	88	$2 \xrightarrow{\beta} 6$ linkages	88
Xanthomonas campestris		glu, man, glu A, acetyl, pyruvate	96	-	
Sarcina ?	-	man	63	1 - 2, 1 - 3, 1 - 6 linkages	63
Alcaligenes faecalis var. mxyogenes	-	glu, gal, man, pyruvate	92	-	
Agrobacter radiobacter	-	glu	103	-	
A. tume-faciens	-	glu	103	-	

Organism	Sero-type	Sugar Composition of Polysaccharide	Reference	Structural details	Reference
Myco-bacterium tuber-culosis	-	man, glu, P	104	-	
Gram-negative soil bacterium	-	D-rham, D-talomethylose	60,61	-	
Unidentified coccus	-	$NAcGNH_2$, glu A	107	$NAcGNH_2$ $1 \xrightarrow{\beta} 3$ Glu A $1 \xrightarrow{\beta} 4$ repeating unit	107

many workers, which use as a measure of effectiveness, the removal of nitrogeneous material. It may be that in some instances protein and other components are integral parts of the molecule, but are removed by the "purification" steps.

In many instances the reports in the literature do not go beyond the details of the sugar composition of the polymer, and outwith the pathogenic species there is very little information on structural details, or any attempt to correlate other results, serological data, or taxonomic relationships, although in one instance, the sugar composition of the extracellular polysaccharides of the genus Rhizobium have been examined, with a view to establishing taxonomic relationships within the group and closely related groups (Graham, 1965). Most information is available about organisms which produce capsules or slime under all conditions of growth, and in particular the demands of medical microbiology have again resulted in several groups of encapsulated pathogenic bacteria being investigated, notably Klebsiella and Pneumococcus. Most or all bacteria from these groups are encapsulated when first isolated, and the organisms have been classified serologically, on the basis of their capsular antigenicity. Attempts to correlate serological reactivity with chemical structure have been made within these three groups, in the same way that the chemical structures of the Salmonella O-antigens have been correlated with serologically determinant groups. Table 4 lists the sugar composition and structural details of various bacterial extracellular poly-

FIGURE 23 STRUCTURE OF K. AEROGENES TYPE 54 EXOPOLYSACCHARIDE



saccharides, in particular the serotypes of the Klebsiella, Escherichia and Pneumococcus groups. Great gaps in knowledge still exist, and there is by no means a comprehensive survey of serological groups and concomitant chemical structures.

Most of the structural details have been elucidated from the examination of oligosaccharides produced by partial hydrolysis of the polysaccharides, and a recent examination has revealed the limitations of this method. Fragments obtained from the extracellular polysaccharide of Klebsiella type 54 (Sandford and Conrad, 1966; Conrad, Bamburg and Kindt, 1966) suggested that the repeating unit of the polymer was as illustrated in Figure 23. Klebsiella strains are known to be hosts for certain phages which produce enzymes capable of depolymerising the extracellular polysaccharide, without destroying its serological reactivity, suggesting release of only large molecular weight fragments (Humphries, 1958; Adams and Park, 1956). Recently Sutherland (1967) obtained a bacteriophage active on type 54 which produced an enzyme capable of hydrolysing the type-specific polysaccharide to give small fragments. Amongst the fragments were oligosaccharides similar to those obtained by partial acid hydrolysis, but of different chromatographic and electrophoretic mobility, and Sutherland has proposed that the repeating unit proposed by Conrad (Conrad, Bamburg and Knat, 1966) is incomplete, O acetyl groups being present, and probably another labile group not yet identified. O acetyl groups are known to be important as antigenic determinants, since Pneumo-

coccal type I loses 34% of its antigenicity on the basis of precipitative power, when such groups are removed (Heidelberger et al., 1950) and similar results have been obtained with E. coli type specific polysaccharides (Hungerer et al., 1967). Obviously many of the structures obtained by relatively drastic procedures, may be lacking labile groups like O acetyl, and the advantages of an enzymic method for polysaccharide breakdown are apparent.

The biosynthesis of bacterial extracellular polysaccharides has been accomplished in only a few instances, despite the large numbers of mucoid organisms. In Acetobacter xylinium, cellulose is produced as a thick pellicle in the growth medium, and Glaser (1958) has prepared an insoluble enzyme system, capable of catalysing the transfer of glucose from UDPG to form a water soluble, alkali insoluble polymer, identified by its sensitivity to cellulase, and formation of characteristic cellulose oligosaccharides on hydrolysis. When the system was treated to remove endogenous polysaccharide material, cellodextrins acted as primers. This system is interesting in view of the apparent involvement of UDPG as the donor, in contrast to the plant world where GDPG has been implicated (Barker, Elbein and Hassid, 1964). The Acetobacter xylinium system has been investigated by another group of workers (Khan and Colvin, 1961a; 1961b) who produced evidence for another precursor. By ethanol extraction of the cells, they obtained a compound, which, when incubated with the soluble fraction from the cells, formed fibrils which were

insoluble to alkali and gave rise to glucose on hydrolysis. The compound was tentatively identified as a glucolipid, remarkable in the light of the present knowledge of lipid-linked intermediates in polysaccharide synthesis. Very little further progress has been made to date, although Glaser's work has been repeated and expanded (Ben-Hazzim and Ohad, 1965) showing that the first stage in the biosynthesis appeared to be the formation of soluble cellodextrin, which subsequently crystallised into fibrils, cocrystallisation with various soluble polysaccharides added to the system taking place. On the basis of these results, the possible role of polysaccharides in the orientation of cellulose fibres in plant cell walls, is discussed. No further evidence has been brought forward for the glucolipids of Khan and Colvin, but their involvement is an attractive hypothesis, when consideration of cell wall permeability is made, and the essentially insoluble wall polymers of bacteria compared with an insoluble polymer such as cellulose. There is one report of the synthesis of a glucan by a particulate system derived from Rhizobium japonicium, in which a polymer, identical with that found normally in the culture medium, was synthesised from UDPG (Doudonder and Hassid, 1964).

Most of the other information on extracellular polysaccharide biosynthesis derived from the system studied in Pneumococcus. A partially fractionated enzyme system from type III incorporated glucose and glucuronic acid from the

corresponding uridine nucleotides, into a polymer which reacted with type III antiserum, and which appeared to have about the same molecular weight as the naturally occuring polymer (Smith et al., 1960). The particulate system could be treated with a specific depolymerase enzyme for the polysaccharide, and the activity of the system appeared to depend on the amount of endogenous polysaccharide material which presumably acted as acceptor (Smith, Galloway and Mills, 1961). On removal of most of the enzymic activity, the system could be markedly stimulated by the addition of oligosaccharide from the polymer containing 8-12 sugar units, smaller or larger oligosaccharides being ineffective (Smith and Mills, 1962), an interesting example of primer requirements. Type I cells produce a rather more complex polysaccharide containing galactose, fucose, galacturonic acid and N-acetyl glucosamine, the structural details being largely unknown (Smith, Galloway and Mills, 1961). Particulate fractions from type I will catalyse the formation of a polymer composed of galacturonic acid from UDPGalA, which reacts with type I antiserum. Addition of UDPNAcGNH$_2$ to the system results in a 100% increase in reactivity with antiserum, suggesting that the naturally occurring polysaccharide has a backbone of polygalacturonic acid, the other sugars being present in side chain (Smith and Mills, 1962). A similar particulate preparation from type VIII cells incorporates glucose, galactose and glucuronic acid from uridine diphosphate derivatives into material precipitable by type VIII antiserum,

and giving the three sugars on hydrolysis (Mills and Smith, 1962c). Recently the biosynthesis of type XIV has been investigated (Distler and Roseman, 1964), the incorporation of galactose, glucose and N-acetyl glucosamine being observed into material which was reactive with antiserum. However, the polymerised material was not identical with the naturally occurring polysaccharide, since the ratios of the sugar components was different. The same fraction catalysed the synthesis of two glycolipids (Kaufmann et al., 1965) which were identified as a glucolipid, and a galactosyl-glucolipid, the possible relationship between these and lipid-linked intermediates in polysaccharide biosynthesis being discussed. However, such lipids are known to occur in a wide range of Gram-positive bacteria, and they have been assessed as a taxonomic tool (Shaw and Baddiley, 1968), no evidence yet being available to suggest their implication in polysaccharide synthesis.

The purpose of the present investigation was to study the occurrence and biosynthesis of a bacterial extracellular polysaccharide which has been said to be common to all forms of mucoid Enterobacteriaceae. The evidence for this generalisation is fragmentary and dates back to the serological studies of Kauffmann (1935; 1936) on the Salmonellae, when a common serological factor in all mucoid forms was observed, the so-called M or mucus antigen. The observations of Kauffmann were repeated by Henricksen (1949; 1950) who showed that mucoid

Escherichia coli also appeared to possess this antigen. Anderson (1961) and Anderson and Rogers (1963) further reported that a large number of the Salmonella-Escherichia group, not normally mucoid, could be induced to form an extracellular slime polysaccharide if grown at 15-20° in high phosphate concentration. The composition of this material appeared to be uniform, containing glucose, galactose, glucuronic acid and fucose. Previous to this, such a polysaccharide had been reported to be produced by mucoid mutants of E. coli K12 (Beiser and Davis, 1957), and Wust (1958) obtained similar mutants. Goebel (1963) isolated a similar polysaccharide from another strain of E. coli, along with a type specific K or capsular antigen, which was quite distinct. Goebel has called the material Colanic acid, and has described it as consisting of 17-20% glucuronic acid, 33-34% galactose, 16-17% glucose and 30-32% fucose, and further went on to isolate antigenically similar material from mucoid mutants of E. coli K12 (Sapelli and Goebel, 1964). Ørskov et al. (1963) have shown a Colanic acid type polysaccharide in three other E. coli strains, one of which had a type-specific K antigen also, which was readily separable by Cetavlon precipitation. In general terms the inference is that members of the Enterobacteriaceae not normally mucoid, such as the Salmonellae, may be induced to produce extracellular polysaccharide of the Colanic acid type, given the correct conditions, and that those members of the group already possessing a type specific capsular antigen, also

produce Colanic acid. On the basis of this it has been suggested that Colanic acid may be the M or mucus antigen of Kauffmann (Sapelli and Goebel, 1964; Lüderitz, Jann and Wheat, 1968; Sharon, 1966).

Markowitz (1964; Markowitz and Rosenbaum, 1965) has investigated the production of Colanic acid in E. coli K12, and has shown that polysaccharide synthesis is controlled by a regulator gene designated CapR which was closely linked to the Pro locus on the bacterial chromosome. The product of this regulator gene appeared to control several enzymes believed to be involved in the synthesis of Colanic acid, such as UDPG-4-Epimerase and GDPFucose synthetase. Heterozygotes produced by transduction showed that the non-mucoid condition was dominant when $CapR^+$ was on the bacterial chromosome, but that when $CapR^+$ was on an episome, the mucoid condition was dominant. Later work (Kang and Markowitz, 1965; 1966) indicated that the product of the regulator gene could be interfered with by growing cells in the presence of p-fluorophenylalanine, non-mucoid cells becoming mucoid, a concomitant increase in enzymes involved in polysaccharide synthesis being observed. Presumably the protein of the repressor produced by the regulator gene is sufficiently altered to allow derepression of certain enzymes. It may be that strainsmucoid under all conditions, are operator constitutive mutants of the type proposed by Jacob and Monod (1961).

The structure of Colanic acid is as yet largely unknown.

FIGURE 24 POSTULATED STRUCTURE OF UNKNOWN SUGAR IN COLANIC ACID

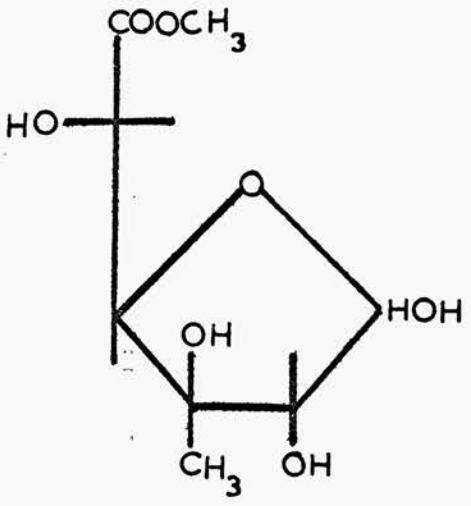


FIGURE 25 STRUCTURAL DETAILS OF COLANIC ACID

X — unknown sugar

Ørskov et al. (1963) have isolated the disaccharide glucuronosyl galactose, later proved to be 3-O-β-D glucuronosyl galactose (Roden and Markowitz, 1966) and Sutherland (unpublished results) has found that the polysaccharide also contains a β glucosyl fucose linkage. Sutherland and Wilkinson (1964) have reported the isolation of bacteriophages which produce enzymes capable of depolymerising the polysaccharide, but unfortunately no small molecular weight fragments were obtained, and the action of the enzymes on the polymer is unknown. Very recently McCleary (1967) has shown some aspects of the structure of Colanic acid, in particular producing evidence for another sugar component, previously undetected. This particular sugar has the postulated structure outlined in Figure 24, being extremely labile to hydrolysis, and only detectable after methylation of the polysaccharide followed by hydrolysis, or by means of β elimination studies. McCleary has suggested an incomplete structure for the polymer (Figure 25), but points out that the repeating unit may not be at all simple, aspects such as the presence of glucuronic acid and glucose units in the main chain having to be accounted for.

MATERIALS AND METHODS

BACTERIAL STRAINS

(a) Escherichia coli K12 strains - previous designations in parentheses.

S5 (K) obtained from the Microbial Genetics Research Unit (MGRU), Hammersmith

S7 (P678) $F^-(\overline{\lambda})$ thr^- leu^- thi^- T_1^R lac^- gal^- str^R MGRU

S8 (58/161) F^- met^- str^R (λ) MGRU

S22 (AB 735) Hfr mal_5^- T_1^R T_6^R λ^S (P_1) Str^S xyl^+ obtained from Dr E.A. Adelberg, Department of Bacteriology, University of California

S23 (AB 739) F^- pro^- his^- try^- arg^- lac^- gal^- T_1^S T_6 Str^R Adelberg

S33 F^- thr^- leu^- thi^- str^R MGRU

S45 (AB 712) F^- pro^- lac^- gal^- thi^- str^R thr^- leu^- Adelberg

S53 Selected from Adelberg strain AB 311 for its chloramphenical resistance by Dr E. Reeve, Department of Animal Genetics, Edinburgh

S53 C Capsular variant of S53. Obtained by Dr I.W. Sutherland, Department of General Microbiology, Edinburgh, during phage resistance studies

S56 (AB 312) Hfr thi^- str^R lac_4^- thr^- leu^- Adelberg

S61 Derivative of S53 producing highly viscous, rubbery colonies, obtained by Dr I.W. Sutherland, Department

of General Microbiology, Edinburgh, isolated by its resistance to a bacteriophage active on S53

CA10 HfrC UDPG pyrophosphorylase-less, obtained from Professor S. Brenner, Laboratory of Molecular Biology, Cambridge

CA 3 HfrC UDPG-4-Epimerase-less Brenner

(b) Wild-type smooth Salmonella strains isolated from pathological material and obtained from the Department of Bacteriology, Edinburgh Medical School.

S. typhi	S. arion
S. paratyphi B	S. braenderup
S. bareilly	S. tubingen
S. heidelberg	S. stanley
S. cholerae var Kunsdorf	S. swartzengrund
M 28247	S. montivideo
S. worthing	S. potsdam
S. brederey	S. enteriditis
S. st. Paul	S. blackley
S. anatum	S. poona

(c) Shigella flexneri NCTC 9725, obtained from Dr D.A.R. Simmonds, Department of Bacteriology, St. Andrews.

Shigella flexneri NCTC 8522 Simmonds

Shigella flexneri Rb mutant of NCTC 9725 Simmonds

Shigella flexneri Ra mutant of NCTC 8522 Simmonds

Aerobacter cloacae NCTC 5920

S. typhimurium NCTC 1098

S. typhimurium LT2 try$^-$ Departmental strain

S. typhimurium SL 1542 his$^-$ Ra mutant obtained from Professor B.A.D. Stocker, Department of Microbiology, Stanford

S. typhimurium SL 1543 mucoid variant of SL 1542 Stocker

Klebsiella aerogenes A4 The strain used by Wilkinson, Duguid and Edmunds (1954)

Klebsiella aerogenes type 54 The strain used by Dudman and Wilkinson (1956)

Klebsiella type 1 obtained from Dr C.H. Clarke, Department of Animal Genetics, Edinburgh

BACTERIOPHAGE STRAINS

All strains were provided by Dr I.W. Sutherland, Department of General Microbiology, Edinburgh.

F_1, F_5, F_{27}: all isolated on E. coli strain S53 as exopolysaccharide depolymerase producers

F_{13}: isolated on Aerobacter cloacae 5920 as an exopolysaccharide depolymerase producer

MEDIA

All basic media were sterilised by autoclaving at 15 lb./sq.in. (121^o) for 15 min. Supplements were sterilised separately, either by autoclaving, or by membrane filtration,

and added aseptically to the medium.

(a) Nutrient broth

Nutrient broth was prepared by dissolving 25 g. Oxoid No. 2 Nutrient Broth Powder (Oxoid Ltd., Southwark Bridge Road, London) in 1 l. distilled water.

(b) Nutrient agar

Nutrient agar was prepared by dissolving 15 g. Oxoid No. 2 Ion Agar in 1 l. nutrient broth.

(c) Minimal A medium

The medium described by Davis and Mingioli (1950) was used, where necessary solidified by the addition of 1.5% (w/v) Ion Agar.

(d) Minimal B medium

The medium described by Davis and Mingioli (1950) was used, supplemented with threonine, leucine, methionine, proline, histidine, tryptophane, arginine and thiamin, which were made up as a separate solution, sterilised by membrane filtration, and added to give a final concentration of 0.002% (w/v). Where necessary the medium was solidified by the addition of 1.5% (w/v) Ion Agar.

(e) Minimal A and B PFA media

The above minimal A and minimal B media were used, supplemented with DL-p-fluorophenylalanine as described by Kang and Markowitz (1966), which was sterilised separately by membrane filtration, and added to give a final concentration of 8×10^{-5} M in liquid media, and 5×10^{-6} M in solid media.

(f) Eosin methylene blue (EMB) agar

EMB sugar media contained (g./l. medium): Oxoid bacto-peptone, 3.0; K_2HPO_4, 0.6; eosin yellow, 0.4; methylene blue, 0.065; Ion Agar, 15.0. The components were dissolved in 900 ml. distilled water by steaming, and the pH adjusted to neutrality with 1.0 N NaOH or 1.0 N HCl. After sterilisation, the basic medium was supplemented with 100 ml. of sterile 10% (w/v) solutions of the appropriate sugar.

(g) Yeast Extract (YE) medium

This nitrogen-deficient was the same as described by Sutherland and Wilkinson (1965). Where necessary the medium was solidified by the addition of 1.5% (w/v) Ion Agar.

BUFFERS

(a) Tris-HCl

A 1.0 M solution of Tris (hydroxymethyl) amino-methane was made up, and brought to the desired pH by the addition of conc. HCl. This buffer was stored at 4^{o}.

(b) Glycine - NaOH

A 1.0 M solution of glycine was made up and brought to the desired pH by the addition of 10.0 N NaOH. This buffer was stored at 4^{o}.

(c) Cysteine - NaOH

A 0.1 M solution of cysteine-HCl was made up and brought to the desired pH by the addition of 1.0 N NaOH. This buffer

was unstable and freshly prepared each time.

(d) Phosphate

0.02 M phosphate buffer, pH 7.2, was prepared according to the method outlined in "Handbook of Bacteriology", ed. Mackie and McCartney, Tenth Edition, Livingston Press.

(e) Saline

Unless otherwise stated, "saline" refers to 0.85% (w/v) NaCl solution in distilled water.

AMINO ACIDS, VITAMINS

The various amino acids, vitamins and analogues of amino acids used were obtained from commercial sources. L-isomers of amino acids were used wherever possible, otherwise DL-compounds were used.

MUTAGEN TREATMENT

(a) Old broth culture

A single colony of the strain to be tested was used to inoculate 100 ml. nutrient broth in a tightly stoppered 100 ml. medical flat. Incubation at 37° was carried out for 3 weeks, and one loopful was spread over the surface of a plate of suitable medium.

(b) Acriflavine

Acriflavine was obtained from British Drug Houses, Ltd., Poole, Dorset. Stock solutions containing 1,000 µg./ml. in

water were autoclaved, and stored in the dark for up to one week. An overnight broth culture of the strain to be treated was diluted to 10^4 cells/ml. in broth containing 100 ug./ml. of acriflavine, and incubated at 37° for 24 hr. Samples were then withdrawn, diluted in saline as necessary, and plated on a suitable medium.

(c) Ethyl methane sulphonate

Ethyl methane sulphonate (EMS) was obtained from Kodak Ltd., Kirkby, Liverpool, and was used as described by Loveless and Howarth (1959). An overnight culture was centrifuged, and the cells were resuspended in an equal volume of 0.4 M EMS for 15 min. at 37°. After further centrifugation the cells were resuspended in broth and incubated at 37° for 16-18 hr. Samples were then withdrawn, and plated on a suitable medium.

(d) Manganous chloride

A modification of the method described by Holloway (1955) was used. Centrifuged cells from an overnight broth culture were suspended in 0.04% (w/v) $MnCl_2$ at a concentration of 10^5 cells/ml., and incubated at 37° for 1 hr. After further centrifugation, the cells were resuspended in nutrient broth and incubated at 37° overnight, before plating on a suitable medium.

PENICILLIN SELECTION TECHNIQUE

The method used by Gorini and Kaufman (1960) was used.

In this method penicillin treatment is carried out in hypertonic Minimal A medium, to prevent the bursting of spheroplasts, thereby minimising cross-feeding of mutants.

A culture of S53 was treated with one of the mutagens mentioned above, and then incubated in broth for 12 hr. at 37° to allow phenotypic expression of mutants. After incubation, a 5 ml. aliquot of the culture was removed, centrifuged, and washed twice with Minimal A medium. A small portion of the pellet was removed, and used to inoculate 5 ml. of Minimal A medium with either glucose, mannose or galactose, as the carbon and energy source, containing 20% (w/v) sucrose, in a 250 ml. Erlenmeyer flask. The flask was shaken vigorously for 2 hr., and then freshly prepared penicillin (Crystapen, Benzylpenicillin B.P., obtained from Glaxo Ltd., Greenford, Middlesex) was added to a concentration of 1,000 i.u./ml. The mixture was then incubated without shaking for a further 3 hr. After this time, the culture was centrifuged, the organisms resuspended in Minimal A medium, and plated on EMB glucose, EMB galactose, or EMB mannose accordingly.

MEMBRANE FILTRATION

Millipore filters (Millipore U.K. Ltd., Heron House, 109 Wembley Hill Road, Wembley, Middlesex) of pore size 0.45 μ or 0.22 μ were used, and filtrates were checked for sterility before use. Swinney hypodermic adaptors (Millipore Ltd.)

were used for small volumes (up to 20 ml.). Larger volumes were passed through a 300 ml. Millipore pyrex filter unit.

CONJUGATION

An overnight broth culture of S22 was diluted 1/10 (v/v) with fresh sterile broth, and incubated for 1-2 hr. at 37°. To a tube containing 1 ml. sterile broth at 37° was added 1.9 ml. from an overnight broth culture of S23, and 0.1 ml. S22. Incubation was continued for 1½-2 hr., when 0.1 ml. of the mixture was spread on plates of Minimal A medium supplemented with 0.002% (w/v) tryptophan, and 1,000 µg./ml. streptomycin (Streptomycin Sulphate B.P., Glaxo Laboratories Ltd., Greenford, England).

CENTRIFUGATION

Small volumes of bacteria in test-tubes, or 1 oz. vials, were centrifuged from culture in an MSE Minor bench centrifuge at a dial setting corresponding to 2,000 g.

Where larger volumes, or higher centrifugal forces were required, centrifugation was carried out in an MSE Highspeed 18 centrifuge or an MSE Superspeed 40 centrifuge. Unless otherwise stated, angle heads were used and the centrifugal force quoted is the maximum obtained at the tip of the tube.

ULTRASONIC DISINTEGRATION

Cells were disrupted in 10-20 ml. batches, cooled in crushed ice, using an MSE 100 watt ultrasonic disintegrator, automatically tuned to 20 Kc/sec.

ISOLATION OF DEPOLYMERASE-PRODUCING BACTERIOPHAGES

(a) Isolation of bacteriophages active on S53

With one exception, the source of all the phages isolated was untreated sewage from Edinburgh sewage works. The technique of Sutherland and Wilkinson (1965) was used, except that membrane filtration rather than chloroform sterilisation was used for the sterilisation of sewage, since it was found that this gave a much higher yield of bacteriophage. Broth lysates of phages in pure culture were membrane filtered and kept at -20°. In one instance, a bacteriophage was picked up as a contaminant of liquid cultures, and this phage was isolated from culture supernates by the same technique.

(b) Detection of depolymerase

Plates of YE medium were uniformly seeded with overnight broth cultures of S53 and allowed to dry. Phage lysates were spotted on, and the plates incubated at 30° for 48 hr. A thick, confluent layer of exopolysaccharide is produced by S53 under these conditions, and those phages producing large haloes round zones of lysis, suggesting depolymerisation of polysaccharide, were selected.

PREPARATION OF EXOPOLYSACCHARIDE DEPOLYMERASES

2-3 hr. shake cultures of S53 in liquid YE medium (250 ml. in 500 ml. Erlenmeyer flasks) were inoculated with 2-3 ml. phage lysates, and incubated overnight at 30° with gentle shaking (100 rev./min.). Any remaining cells were centrifuged at 20,000 *g*, and the supernate dialysed against frequent changes of distilled water at 5° for 4 days. Concentration of the supernate was achieved with polyethylene glycol (M.W. 6,000) according to the method of Kohn (1959). These preparations were kept at -20° without further purification.

Enzymic activity was tested on cultures of S53 by the method of Sutherland and Wilkinson (1965), drops of depolymerase preparation being placed on confluent cultures of various strains, incubated at room temperature for 18 hr., and the plates examined for dissolution of the exopolysaccharide.

PREPARATION OF EXTRACELLULAR (EXO) POLYSACCHARIDES

Bacteria were normally grown on the solid medium which gave the maximal exopolysaccharide production. This proved to be YE medium in most instances, but other media were used. The medium was poured into sterile enamel trays (40 x 30 cm.) as described by Sutherland and Wilkinson (1965). After uniform inoculation from broth cultures, sterile aluminium covers were fitted, and the trays incubated.

The exopolysaccharide slime was scraped off after

incubation, residual traces being washed off with saline, and the resultant viscous solution considerably diluted with saline containing 0.5% (v/v) formaldehyde. Bacterial cells were removed by centrifugation at 23,000 g, and the supernate dialysed against running tap water for 4-7 days. The dialysate was concentrated in vacuo on a rotary evaporator at 60°, and precipitated in acetone, previously cooled to -20°, according to the method of Dudman and Wilkinson (1956). The precipitated material was washed with acetone, dried, redissolved in water, and deproteinised by shaking with chloroform/butanol 5/1 (v/v), the aqueous layer being treated four or five times with fresh chloroform/butanol each time. The aqueous layer was then centrifuged at 20,000 g to remove any precipitate, and freeze-dried. Such preparations have a nitrogen content < 0.5% (w/w) and keep at room temperature in air tight containers.

PREPARATION OF LIPOPOLYSACCHARIDES (LPS)

(a) General method

Cells were grown up overnight at 37° in 500 ml. or 1.5 l. Erlenmeyer flasks of broth. The flasks were half-filled, ensuring adequate aeration, and were shaken on an orbital shaker at 230 rev./min. (Gallenkamp, Technico House, Christopher Street, London). After incubation the cells were centrifuged at 5,000 g, washed twice with saline, and freeze-dried. Pathogenic strains were made safe by the addition of phenol to a

concentration of 10% (w/v).

The LPS were extracted from the dried cells by the phenol-water method (Westphal, Lüderitz and Bister, 1952) and purified at 100,000 g according to the method of Kauffmann et al. (1960), the final preparation being freeze-dried. Preparations of this type were contaminated with a small amount of ribonucleic acid (RNA) as evidenced by a slight absorption at 260 mu, and the appearance of a ribose spot in chromatograms of hydrolysates.

(b) Preparation of RNA-free LPS

Cells were grown in broth as above, centrifuged, and washed twice with saline. The cell paste was resuspended in a small volume of distilled water and subjected to ultrasonic disintegration for 10-15 min., or frozen and thawed four or five times in dry ice/acetone. The debris was removed by centrifugation at 20,000 g, and washed twice with 2.0 M NaCl. The final pellet was freeze-dried and subjected to the phenol-water procedure.

Alternatively, a 10 mg./ml. solution of LPS obtained by the general method was made up in 0.02 M phosphate buffer pH 7.2. Crystalline ribonuclease was commercially obtained, and the solution of LPS treated with 1 mg./ml. ribonuclease for several hours at room temperature. The mixture was then heated at 100° for 90 sec., the precipitated protein centrifuged at 1,000 g, and the supernate dialysed against running tap water for two days. This material was then freeze-dried.

(c) Preparation of lipid-free polysaccharide from LPS

After the method of Davies (1955), a 1% (w/v) solution of

LPS was made up in 1% (v/v) acetic acid, sealed in an ampoule, and hydrolysed at 100° for 1 hr. The precipitated lipid was centrifuged at 1,000 g, the supernate dialysed against running tap water overnight, and then freeze-dried.

CHROMATOGRAPHIC PROCEDURES

(a) Chromatographic solvents

Solvent A pyridine/butanol/water 4/6/3 (v/v) (Whistler and Conrad, 1954)

Solvent B pyridine/ethyl acetate/acetic acid/water 5/5/1/3 (v/v) (Fischer and Dörfel, 1955)

Solvent C ethyl acetate/acetic acid/formic acid/water 18/3/1/4 (v/v) (Feather and Whistler, 1962)

Solvent D Saturated $(NH_4)_2SO_4$/1.0 M sodium acetate, pH 7.5/ isopropanol 80/12/2 (v/v) (Randerath, 1962)

Solvent E 1.0 M Ammonium acetate/ethanol 3/7 (v/v) (Paladini and Leloir, 1952)

Solvent F Chloroform/methanol/water 65/25/4 (v/v) (Weiner et al., 1965)

(b) Electrophoresis buffer

Buffer A pyridine/acetic acid/water 5/2/43 (v/v) pH 5.3 (Sutherland, Lüderitz and Westphal, 1965)

PREPARATION OF HYDROLYSATES OF LPS AND EXOPOLYSACCHARIDES

Exopolysaccharide preparations were hydrolysed in 98/100%

(w/v) formic acid in sealed ampoules for 18 hr. at 100°. The formic acid was then removed by freeze-drying, and the residue rehydrolysed for 2 hr. with 1.0 N H_2SO_4 at 100° to remove formyl esters. The solution was then diluted with distilled water, and neutralised with Amberlite IRA-410 (HCO_3^- form) resin. The resin was removed by filtration, and the solution concentrated to small volume.

LPS preparations were hydrolysed in 1.0 N H_2SO_4 in sealed ampoules for 4 hr. at 100°. The solution was then neutralised and concentrated to small volume as previously described.

Ascending chromatography of the hydrolysates was carried out on thin-layer plates of 0.3 mm. thickness prepared from MN 300 cellulose (Macherey, Nagel and Co., Düren, Germany) in Solvents A, B and C. Sugars were detected with the alkaline $AgNO_3$ reagent of Trevelyan, Proctor and Harrison (1950).

ANALYTICAL METHODS

(a) Spectrophotometry

All spectrophotometric measurements were carried out in a Zeiss PMQ 2 spectrophotometer (Carl Zeiss, Oberkochen, Würtl, Germany). Silica and glass micro, and semi-micro cells were used.

(b) Counting of radioactivity

Samples were counted in an automatic, ambient temperature, liquid scintillation system (Beckmann Instruments, Ltd., Glen-

rothes, Scotland), with automatic print-out. Aqueous samples were counted in a scintillator consisting of NE 572 "scinstant" scintillator dissolved in dioxane (Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh, Scotland). Non-aqueous samples were counted in an 0.5% (w/v) 2,5-Diphenyloxazole (PPO) solution in toluene (Beckmann Instruments, Ltd., Glenrothes, Scotland.) The efficiency of counting in such systems for ^{14}C was 90-93%.

Radioactivity on TLC plates was determined by scraping off the layer into scintillator vials, and counting silica gel in the toluene-based scintillator, cellulose in the dioxane-based scintillator. Radioactivity on paper was determined by cutting out strips, and immersing these in dioxane-based scintillator. The counting efficiency in such systems for ^{14}C ranged from 60% for paper strips, to 85% for silica gel.

(c) Enzymes and chemicals

The following chemicals were obtained commercially as the free form or as sodium or barium salts. Barium salts were converted to sodium salts by treatment with 10% (w/v) sodium sulphate.

Adenine, Cytosine, Guanine, Thymine, Uracil, Adenosine, Cytidine, Deoxy Thymidine, Guanosine, Uridine, AMP, ADP, ATP, ADPM, ADPG, UMP, UDP, UTP, UDPGal, UDPGA, UDPM, UDPNAcGNH$_2$, GMP, GDP, GTP, GDPM, CMP, CDP, CTP, dTMP, dTDP, dTTP, NAD, NADH, NADP, NADPH, G-1-P, G-6-P, F-6-P, M-6-P, glucose, galactose, fucose, rhamnose, mannose, glucuronic acid,

galacturonic acid, N-acetyl glucosamine, glucosamine, muramic acid.

N-acetyl muranic acid was obtained by acetylating muramic acid with acetic anhydride by the method of Strominger, Park and Thompson (1959), purification being accomplished by electrophoresis at 80-100 mA in Buffer A for 4 hr.

Authentic samples of L-glyceroheptose and 2-keto, 3 deoxy-octonic acid (KDO) were provided by Dr I.W. Sutherland, Department of General Microbiology, Edinburgh.

A sample of the unknown sugar believed to be present in Colanic acid was provided by Dr C.W. McCleary, Department of Chemistry, Edinburgh.

The following enzymes were obtained commercially: G-6-P dehydrogenase, hexokinase, phosphoglucomutase, and phospho-glucose isomerase (Boehringer Corporation, London), UDPG dehydrogenase and ribonuclease were obtained from Sigma Biochemicals, London. Commercial preparations of enzymes suspended in $(NH_4)_2SO_4$ were used without further treatment. Freeze-dried preparations such as UDPG dehydrogenase were made up in distilled water.

The following isotopically-labelled compounds were obtained commercially: uniformly labelled ^{14}C-glucose (3.77 mC/mM), uniformly labelled ^{14}C-UDPG (76 mC/mM), uniformly labelled ^{14}C-UDPGal (5.9 mC/mM), uniformly labelled ^{14}C-UDPGA (24.5 mC/mM) and ^{14}C-methyl labelled methionine (56.8 mC/mM).

(d) Assay methods

For microchemical and enzymic work, constriction pipettes were used (H.E. Pedersen, Copenhagen) and all glassware was cleaned with conc. HNO_3 and glass-distilled water. Total volumes were kept to a maximum of 1 ml.

Total sugar was estimated with the anthrone method of Fairbairn (1953); glucose was determined in hydrolysates with glucose oxidase reagent (The Boehringer Corporation, London); galactose in hydrolysates with galactostat reagent (Worthington Biochemical Corporation, New Jersey); glucosamine in hydrolysates by the method of Strominger, Park and Thompson (1959); N-acetyl glucosamine was determined by the method of Strominger, Park and Thompson (1959), omitting the acetylation step; heptose was determined on unhydrolysed material by a modification of the cysteine-H_2SO_4 method, according to Osborn (1963); rhamnose and fucose were determined on unhydrolysed material by the method of Dische and Shettles (1951); KDO was determined on unhydrolysed material by the thiobarbituric acid method of Waravdekar and Saslaw (1959); phosphorus was determined by the method of Fiske and Subba Row (1925); glucuronic acid was determined on unhydrolysed material by the method of Bowness (1957); protein was determined by the method of Lowry et al. (1951).

The following enzyme assays were performed in semi-micro spectrophotometer cells, following the absorption at 340 mu, after the addition of 100 ul. cell-free extract to the assay

mixture:

Phosphoglucomutase was assayed by a modification of the method described by Najjar (1955).

Assay mixture: 100 µl. 0.1 M cysteine-NaOH pH 8.5; 50 µl. 0.02 M G-1-P; 20 µl. 0.02 M NADP; 10 µl. 1.0 M $MgCl_2$; 500 µl. H_2O; 1 µl. G-6-P dehydrogenase (0.14 units).

Hexokinase was assayed by a modification of the method described by Slein, Cori and Cori (1950).

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 7.5; 25 µl. 1.0 M $MgCl_2$; 20 µl. 0.1 M ATP; 30 µl. 0.2 M glucose; 500 µl. H_2O; 20 µl. 0.02 M NADP; 1 µl. G-6-P dehydrogenase (0.14 units).

Gal-1-P Uridyl transferase was assayed by a modification of the method described by Maxwell, Kurahashi and Kalckar (1962).

Assay mixture: 60 µl. 1.0 M glycine-NaOH pH 8.7; 30 µl. 0.1 M cysteine-NaOH pH 8.5; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NADP; 10 µl. 0.02 M UDPG; 30 µl. 0.01 M gal-1-P; 1 µl. G-6-P dehydrogenase (0.14 units); 2 µl. phosphoglucomutase (0.065 units); 500 ul. H_2O.

Galactokinase was detected by a modification of the method described by Maxwell, Kurahashi and Kalckar (1962).

Assay mixture: 60 µl. 1.0 M glycine-NaOH pH 8.7; 30 µl. 0.1 M cysteine-NaOH pH 8.5; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NADP; 10 µl. 0.02 M UDPG; 20 µl. 0.1 M ATP; 1 µl. G-6-P dehydrogenase (0.14 units); 2 µl. phosphoglucomutase (0.065

units); 500 µl. H_2O.

This detection method assumes the presence of a very active gal-1-P uridyl transferase in the extract.

Phosphoglucose isomerase was assayed by a modification of the method described by Slein (1955).

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 7.5; 20 µl. 0.02 M NADP; 10 µl. 1.0 M $MgCl_2$; 20 µl. 0.1 M F-6-P; 100 µl. 0.1 M cysteine-NaOH pH 8.5; 1 µl. G-6-P dehydrogenase (0.14 units); 500 µl. H_2O.

Phosphomannose isomerase was assayed by a modification of the method described by Slein (1955).

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 8.0; 10 µl. 1.0 M $MgCl_2$; 20 µl. 0.1 M M-6-P; 20 µl. 0.02 M NADP; 100 µl. 0.1 M cysteine-NaOH pH 8.5; 1 µl. G-6-P dehydrogenase (0.14 units); 2 µl. phosphoglucose isomerase (0.78 units); 500 µl. H_2O.

UDPG pyrophosphorylase was assayed by a modification of the method described by Munch-Petersen and Kalckar (1955).

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 7.5; 20 µl. 0.02 M UDPG; 60 µl. 0.05 M PP; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NADP; 1 µl. G-6-P dehydrogenase (0.14 units); 2 µl. phosphoglucomutase (0.065 units); 500 µl. H_2O.

GDPM pyrophosphorylase was detected by a modification of the method described by Munch-Petersen (1962).

Assay mixture: 100 µl. 1.0 M Tris-HCl pH 7.5; 20 µl. 0.01 M GDPM; 20 µl. 0.05 M PP; 10 µl. 0.005 M ADP; 10 µl.

0.2 M glucose; 5 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NADP; 1 µl. G-6-P dehydrogenase (0.14 units); 1 µl. hexokinase (0.28 units); 500 µl. H_2O.

PLATE 1 S53 YEAST EXTRACT MEDIUM 30° 48 hr.

PLATE 2 S23 YEAST EXTRACT MEDIUM 30° 48 hr.

RESULTS

EXOPOLYSACCHARIDE PRODUCTION

Various strains of Escherichia coli K12 had been obtained over the years, primarily for genetic purposes, and some of these were observed to produce copious amounts of exopolysaccharide on many different types of media. Such strains are easily recognised on solid media, forming large, raised, viscous colonies, in contrast with the more commonly encountered small, flat, spreading colonies, characteristic of most of the coliform group. The difference between the two types is illustrated in Plates 1 and 2 which show a typical mucoid strain, S53, and a typical non-mucoid strain, S23.

Mucoid variants of K12 have been reported before (Beiser and Davis, 1957; Wust, 1958; Sapelli and Goebel, 1964; Markowitz, 1964) and the exopolysaccharide has been reported to consist of glucose, galactose, fucose, and glucuronic acid. In view of the reports that other members of the Enterobacteriaceae can produce exopolysaccharide of similar composition given the correct conditions, including lowered temperature of growth (Anderson and Rogers, 1963), and the suggestion that this exopolysaccharide constitutes the M antigen or mucoid antigen of the Enterobacteriaceae (Lüderitz, Jann and Wheat, 1968), various members of the Enterobacteriaceae were tested for their ability to produce exopolysaccharide on different types of media.

Organism	Conditions of Growth							
	Nutrient agar		YE		Minimal A		Minimal B	
	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days
S. anatum	0	0	-	+	+	+	0	0
S. arion	0	0	-	±	+	++	0	0
S. braen-derup	0	0	-	-	-	-	0	0
S. stanley	0	0	-	-	-	-	0	0
S. swartzen-grund	0	0	-	-	-	-	0	0
S. monte-video	0	0	-	-	+	+	0	0
S. potsdam	0	0	±	+	+	+	0	0
S. enteri-ditis	0	0	-	±	+	+	0	0
S. blackley	0	0	-	-	-	-	0	0
S. poona	0	0	-	-	-	-	0	0
S. tubingen	0	0	-	-	-	-	0	0

++ very mixed; + mucoid; ± slightly mucoid; - non-mucoid; 0 not tried.

Organism	Conditions of Growth							
	Nutrient agar		Nutrient agar		YE		YE	
	30° 24 hr		20° 7 days		30° 24 hr		20° 7 days	
Sh. flexneri 8522	-		-		-		-	
S. typhi-murium 1542	-		-		-		0	
S. typhi-murium 1543	-		±		-		±	
S. typhi-murium 1098	++		++		++		++	
S. typhi-murium LT2	±		+		++		++	
S. typhi	0		0		-		±	
S. para B	0		0		-		+	
S. bareilly	0		0		-		+	
S. heidelberg	0		0		-		-	
S. cholerae	0		0		-		+	
M 28247	0		0		-		-	
S. worthing	0		0		-		-	
S. brederey	0		0		-		-	
S. St. Paul	0		0		-		-	

Organism	Conditions of Growth			
	Minimal A		Minimal B	
	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days
Sh. flexneri 8522	-	-	-	-
S. typhi-murium 1542	0	0	-	-
S. typhi-murium 1543	0	0	±	±
S. typhi-murium 1098	++	++	++	++
S. typhi-murium LT2	0	0	+	++
S. typhi	±	+	0	0
S. para B	+	++	0	0
S. bareilly	+	+	0	0
S. heidelberg	±	+	0	0
S. cholerae	+	++	0	0
M 28247	-	-	0	0
S. worthing	-	-	0	0
S. brederey	-	-	0	0
S. St. Paul	-	-	0	0

TABLE 5: EXOPOLYSACCHARIDE PRODUCTION

Organism	Conditions of Growth							
	Nutrient agar	Nutrient agar	YE	YE	Minimal A	Minimal A	Minimal B	Minimal B
	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days	30° 24 hr	20° 7 days
S5	-	-	-	-	0	0	-	-
S7	-	-	-	±	0	0	-	±
S8	++	++	++	++	0	0	+	++
S22	++	++	++	++	0	0	+	++
S23	-	-	-	-	0	0	-	-
S33	±	+	++	++	0	0	±	+
S34	-	-	-	-	0	0	-	-
S45	-	-	-	±	0	0	-	±
S53	++	++	++	++	++	++	++	++
S56	-	-	-	±	0	0	-	-
S61	++	++	++	++	++	++	++	++
CA3	-	-	-	-	-	-	-	-
CA10	-	-	-	-	-	-	-	-
A. cloacae 5920	++	++	++	++	++	++	++	++
Sh. flexneri 9725	-	-	-	-	-	-	-	-

The media used included nutrient agar, as typical of a universally used medium for isolation and growth of such bacteria; a simple synthetic medium, containing salts, an inorganic nitrogen source and glucose (Minimal A), supplemented with growth factors if necessary to allow growth of auxotrophs (Minimal B); and the nitrogen deficient medium of Sutherland and Wilkinson (1965) (YE Medium), which enhances polysaccharide production.

Plates of medium were inoculated from overnight streak plates of the same medium incubated at 30°. The inoculated plates were incubated at 30° for 24 hr., and at 20° for 7 days. After the incubation time had elapsed, the plates were examined for exopolysaccharide production, as visualised by the appearance illustrated in Plate 1.

The results are outlined in Table 5. It must be pointed out that in many instances the production of extracellular polysaccharide is not markedly apparent, even on YE medium which generally enhances polysaccharide production. The results range from the obviously very mucoid strains like S53 (Plate 1) which would be designated ++ in the Table, through some strains like S5 and S56 which never produce much polysaccharide on ordinary media even after prolonged growth at low temperature, to others such as S23 and the two Shigella strains examined which have never been observed to produce even small amounts of polysaccharide on ordinary media. To a large extent, exopolysaccharide production on a particular medium

depends on how the strain has been grown or kept previously. It was found that to maintain the maximum amount of polysaccharide production, it is necessary to keep sub-culturing the strain on the medium which gave best exopolysaccharide production. Frequently, even the most mucoid strains such as S53, if revived from stock cultures on nutrient agar, on subculture did not produce much exopolysaccharide until two or three transfers on YE medium had been carried out. It can also be seen that none of the media used enhanced exopolysaccharide production in all strains, some strains producing exopolysaccharide on all types of media, others on only one or two. Generally it was found that YE medium was the best allround medium for enhancing exopolysaccharide production, most strains producing more exopolysaccharide on this medium. However, some of the Salmonella spp. produced exopolysaccharide only on simple synthetic medium, precisely why this should be so is not clear.

Out of thirteen K12 strains examined, 8 produce exopolysaccharide on one or more types of media, ranging from the copiously mucoid S8, S22, S61, S53,and S33 types, to others such as S7 and S45,which only produce small amounts of exopolysaccharide after long incubation at low temperature. Out of 26 other strains of Enterobacteriaceae examined, 13 produced exopolysaccharide, again ranging from the copiously mucoid like S. paratyphi B, and S. typhimurium 1098, to others such as S. typhi ,and S. heidelberg which only produced small amounts of

exopolysaccharide. The heavily encapsulated Klebsiella group, although a member of the Enterobacteriaceae, have not generally been considered to produce the M antigen, and indeed, despite the many reports of chemical details of the capsular structure from various serotypes, few reports have been made of material which might be of the M-antigen type. However, there is a report that an affiliated group, Aerobacter, may produce the M antigen (Sutherland and Wilkinson, 1965), and one of these strains, Aerobacter cloacae 5920, is included in the investigation.

Examination of all the strains which produce exopolysaccharide, by the India ink negative stain method of Duguid (1951), revealed that the only capsulate strain was S53c, the others producing amorphous slime.

EXAMINATION OF EXOPOLYSACCHARIDES

(a) Chromatographic analysis

In order to ascertain similarities between the exopolysaccharides produced by these strains, exopolysaccharides from a representative number of the mucoid strains were prepared. The polysaccharides were prepared from cultures on solid media giving the best exopolysaccharide production, at the optimal temperature and length of incubation. In particular, those strains which produced small amounts of exopolysaccharide only after long incubation at low temperatures, were examined to

TABLE 6: SUGAR COMPOSITION OF EXOPOLYSACCHARIDES

Organism	Conditions of Growth	Monosaccharides in exopolysaccharide hydrolysate			
		Hexose	Deoxyhexose	Uronic acid	Others
S53	YE 30° 24 hr.	glu, gal	fuc	glu A	-
S53	Minimal A 20° 7 days	glu, gal	fuc	glu A	-
S61	YE 30° 24 hr.	glu, gal	fuc	glu A	-
S 8	YE 30° 24 hr.	glu, gal	fuc	glu A	-
S 8	YE 20° 7 days	glu, gal	fuc	glu A	-
S22	YE 30° 24 hr.	glu, gal	fuc	glu A	-
S33	YE 20° 7 days	glu, gal	fuc	glu A	-
S45	YE 20° 7 days	glu, gal	fuc	glu A	-
S56	YE 20° 7 days	glu, gal	fuc	glu A	-
S. typhimurium 1543	YE 20° 7 days	glu, gal	fuc	glu A	-
S. typhimurium LT2	Minimal B 20° 7 days	glu, gal	fuc	glu A	-
S. paratyphi B	Minimal A 30° 24 hr.	glu, gal	fuc	glu A	-
S. cholerae	Minimal A 30° 24 hr.	glu, gal	fuc	glu A	-
S. enterditis	Minimal A 20° 7 days	glu, gal	fuc	glu A	-
S. typhimurium 1098	YE 30° 24 hr.	glu, gal	fuc	glu A	-
S. bareilly	Minimal A 20° 7 days	glu, gal	fuc	glu A	-

glu - glucose; gal - galactose; fuc - fucose; glu A - glucuronic acid

TABLE 7: QUANTITATIVE ANALYSES OF EXOPOLYSACCHARIDES

Organism	% fucose	% galactose	% glucose	% glucuronic acid
S53	27.1 - 32.0	27.8 - 31.7	12.9 - 15.3	16.8 - 19.7
S53C	29.2 - 34.1	27.8 - 32.1	13.9 - 16.2	16.8 - 18.3
S61	22.7 - 30.0	25.8 - 30.3	12.1 - 16.3	17.2 - 21.0
S 8	25.7 - 33.8	31.0 - 32.8	15.9 - 18.7	15.3 - 19.2
S22	28.1 - 34.0	30.0 - 32.2	16.0 - 18.1	18.0 - 20.1
S33	26.2 - 29.7	28.2 - 29.8	15.2 - 17.0	19.0 - 19.7
S45	25.8 - 29.1	27.3 - 28.2	14.9 - 15.7	17.3 - 18.9
A. cloacae 5920	26.0 - 27.3	25.7 - 29.2	15.0 - 17.3	16.0 - 16.8
S. typhimurium 1543	27.3 - 29.0	30.0 - 31.5	14.7 - 16.3	18.2 - 22.0
S. typhimurium LT2	28.0 - 29.2	29.2 - 33.8	12.9 - 17.2	19.1 - 19.9
S. typhimurium 1098	25.6 - 28.7	27.8 - 31.7	15.3 - 17.1	17.1 - 17.7
S. paratyphi B	22.9 - 26.3	25.3 - 29.1	14.9 - 17.1	15.7 - 16.1
S. cholerae	23.8 - 26.8	24.8 - 27.2	16.1 - 18.7	15.7 - 21.2
S. enterditis	27.1 - 28.7	29.1 - 29.4	19.1 - 21.0	21.7 - 24.0
S. bareilly	28.4 - 31.7	29.1 - 33.8	17.1 - 18.2	21.0 - 22.7

A minimum of four estimations carried out for each sugar.

determine if this exopolysaccharide was different from that produced by some strains under all conditions, on all types of media. The polysaccharide preparations were hydrolysed, neutralised, and analysed on cellulose thin layer in Solvents A, B, and C. Sugars were identified on the basis of their chromatographic mobilities in all three solvents, compared with the standard sugars available.

The results are outlined in Table 6. As can be seen, all of the exopolysaccharide preparations examined had the same chemotype, containing only glucose, galactose, fucose and glucuronic acid, the sugars found in Colanic acid described by Goebel (1963), and the slime polysaccharides described by Anderson and Rogers (1963).

(b) Quantitative analysis of sugar components

Goebel (1963) described Colanic acid as containing 17-20% glucuronic acid, 33-34% galactose, 30-32% fucose, and 16-17% glucose. In order to ascertain if the exopolysaccharides examined had the same quantitative composition as Colanic acid, sugar analyses were carried out on several of these, on un-hydrolysed and hydrolysed preparations. In Table 7, the percentage compositions are shown. It can be seen that the analyses vary quite appreciably in some instances, the lowest and highest figure of a series of measurements being shown in the Table. The variations reflect the limitations of the assay methods employed. The fucose and glucuronic acid

estimations, performed on intact polysaccharides, are particularly subject to fluctuation, and, while the enzymic estimations of free glucose and galactose are extremely accurate, the limitation when applied to this system is the hydrolysis of the exopolysaccharide, different hydrolysates giving different results, a reflection of the sugar destruction in each case. Presumably these limitations are responsible for the fact that in very few instances would the sugars determined add up to 100%, despite extensive drying of the polysaccharide to eliminate hydration, and correction for nitrogen content. Nevertheless, the values obtained correspond well with the figures quoted by Goebel (1963) and Sapelli and Goebel (1964), suggesting a Colanic acid type of polysaccharide with the molar ratios of sugars approximating to 2:2:1:1 for fucose, galactose, glucose, and glucuronic acid respectively.

PHAGE-INDUCED DEPOLYMERASES AND EXOPOLYSACCHARIDE IDENTIFICATION

Sutherland and Wilkinson (1965) obtained results which suggested that exopolysaccharide depolymerases, induced by bacteriophages, were active only on exopolysaccharides identical to that of the host cell. It was thought that this might be a reasonable method for identifying exopolysaccharides, in much the same way as certain pathogenic bacteria are typed by virtue of bacteriophage specificity towards groups in the bacterial cell-wall or cell envelope.

Several bacteriophages known to produce depolymerases active on the Colanic acid type of exopolysaccharide were provided by Dr I.W. Sutherland, designated F_1, F_5, and F_{27}, isolated on S53 as host, and F_{13}, isolated on Aerobacter cloacae 5920. Five other depolymerase producing phages were isolated from untreated Edinburgh sewage, on S53 as host, designated B_1, B_2, B_3, B_4, B_5, and a further depolymerase producer, B_6, as a chance contaminant from liquid cultures of S53.

Depolymerase preparations were obtained from phage lysates of these bacteriophages on their host strains, and the specificity of the depolymerase preparations was determined by ascertaining the action of the preparations on several different types of exopolysaccharide. Dudman and Wilkinson (1956) have shown that the exopolysaccharide of K. aerogenes A4 contains glucose, galactose, and glucuronic acid, and is therefore quite different from the Colanic acid type. Similarly, Klebsiella type 54 can be distinguished from the Colanic acid type, since it contains glucose, glucuronic acid, and fucose (Wilkinson, Dudman and Aspinall, 1956). Recently Klebsiella type 1 has been shown to have the same chemotype as Colanic acid (I.W. Sutherland, unpublished results), and is chemically identical as far as is known although structural details have not yet been ascertained.

Depolymerase preparations were tested on old YE cultures of S53, Aerobacter cloacae 5920, Klebsiella types 1 and 54, and

Klebsiella aerogenes A4. The plates were incubated and examined for dissolution of the exopolysaccharide. The results showed clearly that all the depolymerase preparations were active only on the exopolysaccharides of S53 and Aerobacter cloacae 5920. Of particular significance is the insensitivity of the Klebsiella type 1 exopolysaccharide, despite its identical chemotype, indicating that the depolymerase enzymes seem to have a high degree of specificity even within polysaccharides of the same chemotype. In view of the evidence that the exopolysaccharide of mucoid mutants of E. coli K12 is Colanic acid (Sapelli and Goebel, 1964) it seems likely that these depolymerase preparations are quite specific for Colanic acid. An interesting feature is that the F_{13} bacteriophage does not attack K12 cells, and K12 bacteriophages such as F_1 and F_5 do not attack Aerobacter cloacae 5920 cells (Sutherland and Wilkinson, 1965), and yet these bacteriophages produce a common Colanic acid depolymerase. Since the specificity of the depolymerase preparations seemed precise as far as was determined, it appeared valid to use these preparations to type exopolysaccharides produced by the various strains, as affording a more accurate method of determining similarities than chemical estimations.

Those strains of bacteria which produced sufficient polysaccharide on solid media to enable detection of enzymic activity, were tested in the same way with drops of depolymerase preparation spotted on to old lawn cultures of bacteria on the

TABLE 8: EXOPOLYSACCHARIDES TESTED FOR SENSITIVITY TO DEPOLYMERASE PREPARATIONS

Exopolysaccharide preparations from the following strains:

S53	S. typhimurium 1098
S53C	S. typhimurium LT2
S 8	S. typhimurium 1543
S22	S. paratyphi B
S33	S. bareilly
S61	S. cholerae var. Kunsdorf
A. cloacae 5920	S. heidelberg

medium giving best polysaccharide production. The strains are listed in Table 8. In all cases the exopolysaccharides were sensitive to all the depolymerases clearly indicating, when taken into account with the chromatographic and qualitative data, the likelihood that all the exopolysaccharides produced are identical to Colanic acid.

Two strains, S53c and S61, are slightly surprising in this respect. S53c is a capsulate variant of S53, and, although Wilkinson (1958) has said that there can be no definite distinction between capsular and slime material, this viewpoint was largely based on chemical evidence. In the light of the apparently fine specificity of depolymerase preparations, it might have been supposed that the change from slime to capsule involved the alteration of the chemical structure of the polysaccharide in some way undetectable by conventional techniques, and that the sensitivity of the exopolysaccharide to depolymerases might have changed. S61 is also unusual in that the exopolysaccharide is physically entirely different from all of the other strains, being extremely rubbery in texture, despite having all the chemical characteristics of Colanic acid.

p-FLUOROPHENYLALANINE (PFA) AND DEREPRESSION OF EXOPOLYSACCHARIDE SYNTHESIS

Kang and Markowitz (1965) have suggested that in *E. coli* K12 Colanic acid synthesis is controlled by a repressor, which

Organism	Conditions of Growth	
	Minimal medium 20° 7 days	Minimal PFA medium 20° 7 days
S. stanley	-	+
S. swartzengrund	-	-
S. montevideo	+	+
S. potsdam	+	+
S. blackley	-	±
S. poona	-	++

TABLE 9: EXOPOLYSACCHARIDE PRODUCTION IN THE PRESENCE OF PFA

Organism	Conditions of Growth	
	Minimal medium 20° 7 days	Minimal PFA medium 20° 7 days
S 5	-	++
S 7	±	+
S23	-	-
S34	±	++
S45	±	+
S56	±	++
CA3	-	-
CA10	-	-
S. typhimurium 1542	-	-
S. typhimurium 1543	±	±
Sh. flexneri 9725	-	-
Sh. flexneri 8522	-	-
S. typhi	±	+
M 28247	-	+
S. worthing	±	±
S. brederey	-	++
S. st. Paul	±	±
S. arion	+	++
S. braenderup	-	+
S. tubingen	-	+

normally prevents visible synthesis of exopolysaccharide on solid media, resulting in most strains of K12 being non-mucoid. The supposition is that mucoid mutants are derepressed in some way, either by a change in the repressor or the operator. Kang and Markowitz (1965) further went on to show that many non-mucoid strains of K12 can be made mucoid by growing them in the presence of p-fluorophenylalanine, presumably because the PFA is incorporated into the repressor molecule, altering its function in some way.

In the light of this information and the evidence that many other strains of the Enterobacteriaceae appear to produce Colanic acid under certain conditions, it was decided to grow the previously designated non-mucoid strains on solid PFA media, including some of the strains which produced only small amounts of exopolysaccharide. The results are outlined in Table 9, strains being grown on Minimal A or Minimal B PFA medium, depending on growth requirements, at 20° for 7 days. It can be seen that of 8 K12 strains previously thought non-mucoid, or only producing small amounts of exopolysaccharide, 3 produced a large amount of exopolysaccharide when grown in the presence of PFA and the others appreciably more than on ordinary media, the exceptions being CA3, CA10 and S23. Out of the other previously designated non-mucoid species of Enterobacteriaceae tested, 7 became mucoid on PFA, and 3 other strains considered to produce only small amounts of exopolysaccharide normally, elaborated considerably more on PFA. Of these strains tested, those that

TABLE 10: PFA EXOPOLYSACCHARIDE PREPARATIONS TESTED FOR SENSITIVITY TO DEPOLYMERASE ENZYMES

Exopolysaccharide preparations from the following strains:

S 5	M 28247
S 7	S. brederey
S34	S. arion
S56	S. anatum
S. typhimurium 1543	S. poona

TABLE 11: SUGARS IN HYDROLYSATES OF ACETONE-PRECIPITABLE MATERIAL FROM SUPERNATES OF STRAINS GROWN ON LIQUID MINIMAL PFA MEDIUM

Organism	Hexoses	Uronic acid	Deoxyhexoses	Pentoses	Dideoxyhexoses
CA 3	glu	-	-	rib	-
CA10	-	-	-	rib	-
S23	glu, gal	-	rham	rib	-
Sh. flexneri 8522	glu, gal	-	rham	rib	-
Sh. flexneri 9725	glu, gal	-	rham	rib	-
S. typhi	glu, gal	glu A	fuc, rham	rib	+
S. worthing	glu, gal, man	glu A	fuc, rham	rib	+
S. st. Paul	glu, gal	glu A	fuc, rham	rib	-
S. braenderup	glu, gal, man	glu A	fuc, rham	rib	-
S. tubingen	glu, gal	glu A	fuc, rham	rib	-
S. stanley	glu, gal, man	glu A	fuc, rham	rib	+
S. swartzengrund	glu, gal	glu A	fuc, rham	rib	-
S. montevideo	glu, gal	glu A	fuc, rham	rib	-
S. potsdam	glu, gal	glu A	fuc, rham	rib	-
S. blackley	glu, gal	glu A	fuc, rham	rib	-

glu - glucose, gal - galactose, fuc - fucose, rham - rhamnose, rib - ribose

synthesised sufficient polysaccharide were tested with depolymerase preparations, using lawn cultures of strains on solid PFA medium incubated at 20° for 7 days, followed by a further 7 days at 4°. In all cases, the exopolysaccharides tested were sensitive to all the depolymerase preparations, the strains being listed in Table 10, and it seems clear that the exopolysaccharide produced in the presence of PFA is Colanic acid.

The results were sufficiently conclusive to suggest that the other strains producing small amounts of exopolysaccharide also produced Colanic acid, and that those strains such as CA3, CA10, S23, Shigella flexneri and S. swartzengrund, which appeared non-mucoid under these conditions, might produce small amounts of exopolysaccharide, too small in amount to be detected visually. Consequently several strains were grown in liquid Minimal A or B PFA medium at 20° for 7 days, the cells spun off after addition of 0.5% (v/v) formaldehyde, and the supernate concentrated and precipitated in acetone. The acetone precipitable material was deproteinised by shaking with chloroform-butanol, and hydrolysed. The hydrolysates were chromatographed on thin layer cellulose in Solvents A and B, sugars being detected with alkaline silver nitrate. The sugars detected are given in Table 11. As can be seen, with the exception of CA3, CA10, S23 and the two Shigella spp., spots with the chromatic mobility of glucose, galactose, fucose, and glucuronic acid, were detected in all the

hydrolysates. Unhydrolysed material from all these preparations also gave a positive colour reaction for uronic acid with the carbazole assay method of Bowness (1957).

Other sugars were also detected in the material, probably arising from contamination by lipopolysaccharides and nucleic acid, since the long incubation time inevitably results in some cell lysis, particularly since PFA inhibits growth quite considerably. However, taken along with the other results, these results strongly suggest that many strains of Salmonella have the capability of synthesising exopolysaccharide of the Colanic acid type albeit in small amounts.

S. typhi is unique amongst the Salmonellae in that it produces the V_i antigen, an exopolysaccharide microcapsule composed of polyaminohexuronic acid. Clearly such material would give a positive colour reaction in any estimation of uronic acids, and hydrolysis products are likely to move similarly to glucuronic acid in thin layer. However, the presence of fucose in the hydrolysate is probably significant, suggesting the presence of Colanic acid in addition to polyaminohexuronic acid.

The strains CA3 and CA10 were received as UDPG-4-Epimerase-less and UDPG pyrophosphorylase-less mutants of E. coli K12, and since the exopolysaccharide contains galactose and glucose, the inability of these strains to synthesise one or other of these sugars as an uridine diphosphate derivative, and the concomitant inability of either strain to produce exopolysaccharide under

any circumstances, strongly suggests the involvement of UDPG and UDPGal in the synthesis of the exopolysaccharide.

ATTEMPTS TO ISOLATE STRAINS DEFECTIVE IN SOME ASPECT OF EXOPOLYSACCHARIDE SYNTHESIS

In view of the success obtained by various workers, using mutant strains of bacteria in the elucidation of the structure and biosynthesis of LPS, clearly similar mutants blocked in some aspect of exopolysaccharide synthesis, would be of great value in any investigation, and consequently throughout this investigation attempts were made to isolate any apparently mutant organisms.

(a) Since Colanic acid contains glucose, galactose and fucose, similar to many lipopolysaccharides, attempts were made to get mutants which would not ferment the sugars glucose and galactose, in direct analogy with the mutants obtained by many workers in LPS biosynthesis. The inability to ferment these sugars generally indicates a defect in certain key enzymes concerned with glucose and galactose metabolism, and therefore hopefully with exopolysaccharide synthesis. Such mutants have only been obtained with difficulty in the past by various groups, including the glucose-negative, phosphoglucose isomerase-less mutant of Fraenkel et al. (1963), and the galactose-negative, UDPG pyrophosphorylase and UDPG-4-Epimerase-less mutants of Sundarajan, Rapin and Kalckar (1962) and Osborn (1963).

Likewise, phosphomannose isomerase-less mutants have been isolated as mannose-negative colonies (Zeleznick et al., 1965), and since fucose is generally synthesised from a nucleoside diphosphate derivative of mannose, then such mutants would be unable to synthesise fucose in addition to mannose.

The strain S53 was selected as a suitable parent strain since it produces copious amounts of exopolysaccharide on all types of media, and it was subjected to mutagen treatment, including growing cultures in nutrient broth for 2-3 weeks at 37°, $MnCl_2$, acriflavine, and ethyl methane sulphonate treatment. Treated cultures were plated out on EMB glucose, EMB galactose, and EMB mannose, and examined for colonies unable to ferment the sugar. Despite many attempts, no sugar-negative colonies were detected and penicillin selection to enrich for such mutants was also unsuccessful.

However, during these procedures several colonies were observed, which, although having the same sugar reaction as S53, were non-mucoid on EMB media. One isolated from old broth cultures was designated S53/1, one from EMS treated culture S53/2, and one from acriflavine treated cultures S53/3.

(b) The strains CA10 and CA3 are both galactose-negative strains, and the colonies eventually disintegrate on galactose containing media. The strain CA10 was observed to revert occasionally to galactose positive, and one of these galactose positive colonies was picked off and designated CA10 R.

(c) S22 is a highly mucoid strain, and as with other highly

TABLE 12: EXOPOLYSACCHARIDE PRODUCTION BY DERIVATIVES OF VARIOUS K12 STRAINS

Organism	Conditions of Growth						Sensitivity of exopoly-saccharide to depolymerases
	Nutrient agar	Nutrient agar	YE	YE	Minimal medium	Minimal PFA	
	30° 24 hr.	20° 7 days	30° 24 hr.	20° 7 days	20° 7 days	20° 7 days	
Parent S53	++	++	++	++	++	++	+
S53/1	-	-	-	-	-	-	0
S53/2	-	-	-	-	-	-	0
S53/3	-	-	-	-	-	-	0
Parent S22	++	++	++	++	++	++	+
S22M	-	-	-	-	±	++	+
Parent S23	-	-	-	-	-	-	0
S22-23	++	++	++	++	++	++	+
Parent CA10	-	-	-	-	-	-	+
CA10R	-	-	-	-	-	++	+

0 - not tried

mucoid strains had not been observed to revert to non-mucoid during routine plating out procedures. However, an old stock culture which had been kept on a nutrient agar slope for 2 years, when plated out was observed to have a high proportion of flat, non-mucoid, spreading colonies. One of these colonies was purified and the strain designated S22 M.

(d) S22 is also a Hfr strain and can be easily crossed with S23 which is a non-mucoid strain under all conditions of growth. During crosses wild-type streptomycin resistant strains were obtained and a high proportion of such recombinants were very mucoid. One such recombinant was purified and designated S22-23.

EXOPOLYSACCHARIDE PRODUCTION BY CA10 R, S22 M, S53/1/2/3 AND S22-23

The strains were grown on plates of nutrient agar, YE medium, and Minimal A PFA medium or Minimal B PFA medium depending on growth requirements as previously described. The results are outlined in Table 12. It can be seen that the S53 derivatives do not produce exopolysaccharide on any of the media investigated. Further growth in liquid Minimal PFA medium, as previously described, gave acetone precipitable material which did not give a positive colour reaction for uronic acid, and on hydrolysis gave only glucose, galactose, ribose and rhamnose in all cases. The derivatives of S22 and CA10 appear to behave

as typical wild-type K12 strains, having the ability to produce exopolysaccharide, but normally non-mucoid unless derepressed by growing in the presence of PFA. The exopolysaccharide was sensitive to the phage enzymes in both cases. Strain S22-23 appeared to be indistinguishable from S53 or any other mucoid strain, producing copious amounts of exopolysaccharide on all types of media.

It seems likely that CA10 is unable to produce exopolysaccharide under any conditions because of its inability to make UDPG, and that the revertant CA10 R is a normal K12 strain, able to synthesise exopolysaccharide when the necessary enzymes, including those involved in the synthesis of UDPG are derepressed. S22 M is an interesting strain since it appears that a reversion from derepressed to repressed has taken place, since the parent S22 produces large amounts of exopolysaccharide on all types of media.

COMPARISON OF VARIOUS STRAINS BY LPS ANALYSIS

The possibility occurred that many of the strains able to synthesise Colanic acid, already had the necessary precursors in the cell for other reasons such as the biosynthesis of LPS. With this in mind it was decided to examine a representative number of LPS from these strains in order to ascertain any sugar components shared between LPS and exopolysaccharide. Clearly, any shared sugar components need not have been formed

TABLE 13: SUGAR ANALYSIS OF LPS OF VARIOUS STRAINS

Organism	Heptose	KDO	P	Glucose	Galactose	Mannose	$NAcGNH_2$	Deoxyhexose	Dideoxyhexose
S53	+	+	+	+	+	-	+	rhamnose	-
S53/1	+	+	+	+	+	-	+	rhamnose	-
S53/2	+	+	+	+	+	-	+	rhamnose	-
S53/3	+	+	+	+	+	-	+	rhamnose	-
S61	+	+	+	+	+	-	+	rhamnose	-
S56	+	+	+	+	+	-	+	rhamnose	-
S33	+	+	+	+	+	-	+	rhamnose	-
S23	+	+	+	+	+	-	+	rhamnose	-
S22	+	+	+	+	+	-	+	rhamnose	-
S22M	+	+	+	+	+	-	+	rhamnose	-
CA10	+	+	+	-	-	-	-	-	-
CA10R	+	+	+	+	+	-	+	rhamnose	-
CA3	+	+	+	+	-	-	-	-	-
A. cloacae 5920	+	+	+	+	-	-	+	rhamnose	-
S. typhimurium 1098	+	+	+	+	+	+	+	rhamnose	+
S. typhimurium 1542	+	+	+	+	+	-	+	-	-

Organism	Heptose	KDO	P	Glucose	Galactose	Mannose	$NAcGNH_2$	Deoxyhexose	Dideoxyhexose
S. typhimurium 1543	+	+	+	+	+	-	+	-	-
S. typhi	+	+	+	+	+	+	+	rhamnose	+
S. typhimurium LT2	+	+	+	+	+	+	+	rhamnose	+
S. anatum	+	+	+	+	+	+	+	rhamnose	+
Sh. flexneri 8522	+	+	+	+	+	-	+	rhamnose	-

as the same nucleotide derivatives in both cases, but the simultaneous deletion of such a sugar from the LPS and loss of the ability to produce exopolysaccharide would be significant. In particular the strains which were non-mucoid under all conditions of growth such as S23, and the S53 variants, are interesting in this respect.

In addition, the possibility of similarities between the various strains in terms of LPS composition was considered, despite the pathological, biochemical and serological differences which exist between the strains.

LPS preparations were made by the general method (Method (a)), and hydrolysates were subjected to chromatographic analysis. KDO, heptose, and phosphate were detected in unhydrolysed material by their colour reactions, the other sugar components by their chromatographic mobilities on thin-layer cellulose in Solvents A, B, and C. The results are outlined in Table 13. In all cases, ribose was detected, presumably due to contamination of the preparations with RNA. This was confirmed by obtaining LPS preparations from the cell walls of various strains, and treating material obtained by the general method with ribonuclease (Method (b)). In both cases examination of hydrolysates of the material obtained, showed the disappearance of the ribose spot.

In all cases glucosamine was detected chromatographically, and it was thought that its presence was probably largely due to the lipid portion of the LPS which is known to contain

glucosamine. Several LPS preparations were treated by hydrolysis with 1% (w/v) acetic acid to remove the lipid, and chromatographic analysis of hydrolysates showed the glucosamine spot to be greatly diminished in all cases. Complete removal of glucosamine could not be achieved except in the case of strains CA3 and CA10, presumably because, with the exception of these two strains, the LPS of the others contained N-acetyl glucosamine as an additional amino sugar, and acid hydrolysis liberates some of the acetyl groups, giving rise to free glucosamine.

Several of the Salmonella strains appeared to have fast moving sugar components in hydrolysates of LPS. These components ran considerably faster than rhamnose in Solvent systems A, B, and C and are assumed to be 3,6 dideoxyhexoses. It seems likely that the spot encountered in chromatograms of S. typhi LPS hydrolysates is tyvelose, and that in S. typhimurium LPS hydrolysates, abequose.

It can be seen that most of the K12 strains appear to contain glucose, galactose, N-acetyl glucosamine and rhamnose in addition to a polyheptose-Phosphate-KDO backbone. Significantly, strains S53/1, S53/2, S53/3 and S23, which are non-mucoid under all conditions of growth, have the same LPS composition as the mucoid types. Strain CA3 is unable to synthesise UDPGal, and, as might be expected, has a LPS consisting of only the backbone with additional glucose. Strain CA10 is unable to synthesise UDPG and has an even more

incomplete LPS, consisting of only a polyheptose-phosphate-KDO backbone. The revertant CA1O R synthesises the normal K12 LPS with all the additional sugars.

The involvement of UDPG and UDPGal in Colanic acid synthesis seems likely in view of the fact that neither CA3 nor CA1O are able to synthesise the polymer, but CA1O R when grown in the presence of PFA synthesises Colanic acid. Clearly the other non-mucoid strains, such as S23 and S53 variants, are not likely to be non-mucoid by virtue of a defect in the synthesis of UDPG or UDPGal, since these sugars are present in the LPS.

The only sugars shared between LPS and exopolysaccharide in all the strains examined seem to be glucose and galactose, although some of the Salmonellae have mannose in the LPS, and mannose is generally taken to be involved in the synthesis of fucose. Aerobacter cloceae 5920 is unusual in that the LPS does not contain galactose, so that only glucose is found in LPS and exopolysaccharide.

Of the strains other than the K12 types, the only similarities apparent are the possession of glucose, galactose and rhamnose in all cases examined with the exception of S. typhimurium 1542 and 1543. Both of these strains are Ra mutants, synthesising only the core structure of the LPS, by virtue of a defect in the synthesis of one of the sugars in the O-specific region of the LPS. The deficiency is said to be in the pathway leading to the synthesis of the nucleoside diphosphate derivative of mannose, and since S. typhimurium 1543

appears to produce small amounts of fucose containing Colanic acid under certain conditions, this is extremely interesting in view of the evidence to suggest that the nucleoside diphosphate derivative of fucose is synthesised from the corresponding mannose derivative.

PREPARATION OF NUCLEOTIDE EXTRACTS OF STRAINS

The analysis of the nucleotide pool of several strains is clearly important, to ascertain precisely which nucleotide derivatives of the sugars found in Colanic acid are present, in order to give an indication of the enzyme systems involved in its synthesis. There is also the possibility that analysis of the nucleotides of the non-mucoid strains will give an indication of why these are non-mucoid. Of additional interest are the nucleotide pools of those repressed strains which only become mucoid when grown in the presence of PFA, in order to determine at which stage the repressor molecule acts.

Initially, cells were grown in liquid media giving maximal exopolysaccharide production, such as YE medium. However, it was found that under these conditions the cells of mucoid strains produced so much exopolysaccharide that it was almost impossible to remove them by centrifugation. In a large-scale experiment, involving perhaps 20 l. of culture, it became totally impossible, so a medium giving a reasonable, but not excessive yield of exopolysaccharide had to be chosen. In

practice it was found that nutrient broth sufficed in many instances, therefore this medium was most commonly used and the cells were grown in it unless otherwise stated.

(a) Exponential-phase cells

In view of the technical difficulties in obtaining a large quantity of cells in the exponential phase without access to a large fermenter, a non-aseptic technique was used. An overnight shake culture of the organism in 10 l. broth was prepared in Erlenmeyer flasks. A washing machine of 40 l. capacity (Hoover, Ltd.) was filled with 30 l. distilled water, brought to boiling point, covered, and allowed to cool. When the temperature had reached 30-35°, 500 g. Oxoid Nutrient Broth No. 2 was dissolved, followed by the addition of the 10 l. overnight culture. The agitator on the washing machine was started, and turbidity measurements made on the contents at regular intervals. The temperature remained steady at 30-35° without any additional heating, and after 2-3 hr. the cells were adjudged to be largely in the exponential phase. Centrifugation at 0° and 20,000 g was then carried out using a continuous action rotor, at a rate of 500 ml./min. The resultant cell paste was suspended in a small volume of ice-cold saline, and poured into 3 volumes boiling ethanol, extracting with stirring for 10 min. The cell debris was removed by centrifugation at 5,000 g, and the extract concentrated to small volume at 20° on a rotary evaporator.

Deproteinisation was carried out by shaking the extract with an equal volume of chloroform for 10-15 min., followed by centrifugation at 40,000 g, the aqueous layer being removed and kept at -20°.

Several such preparations were achieved with various E. coli K12 strains, until contamination with an extremely virulent bacteriophage occurred. Sterilisation of the equipment with various disinfectants, and removal of the equipment to another building proved ineffective in removing the phage, resulting in the eventual abandonment of the technique.

(b) Stationary-phase cells

Cells were grown overnight in shake culture in half-full Erlenmeyer flasks, the cells being harvested and ethanol extracted as previously described.

ANALYSIS OF NUCLEOTIDE POOLS

Column chromatography of nucleotide extracts was carried out on Whatman ET 11 ECTEOLA cellulose on 25 x 1 cm. or 45 x 1.5 cm. columns by a modification of the method described by Nilsson and Sjunnesson (1961).

1.0 M triethyl ammonium acetate buffer was made up by adjusting a 1.0 M solution of tmethyl amine in distilled water to pH 4.0 or 6.0 with glacial acetic acid.

ET 11 cellulose powder was suspended in 0.5 N NaOH under vacuum for 30 min., then washed on a sintered glass pad under

suction with distilled water until the washings were neutral. The powder was then resuspended in 1.0 M triethyl ammonium acetate pH 4.0 and again degassed under vacuum for 30 min., after which time a column was packed with the cellulose under gravity, and equilibrated with the same buffer at 25 ml./hr. for 24 hr. The column was then washed through with distilled water at the same flow rate for 24 hr., and the nucleotide extract adsorbed on to the top of the column, washing in with a little distilled water.

A buffer gradient system was set up, comprising a 1 l. straight-sided mixer vessel containing 500 ml. distilled water, which was pumped into the top of the column at 25 ml./hr., a buffer reservoir siphoning into the mixing vessel. The effluent from the column was continuously monitored at 254 mu and recorded (LKB 4700 A Uvicord I, and recorder, LKB Instruments Ltd., 137 Anerley Road, London).

Initially water was passed through the column, in order to wash through any uncharged UV absorbing substances. A large peak of such material was quickly eluted, collected and freeze-dried. As soon as the UV absorbance returned to zero, the distilled water in the buffer reservoir was replaced with 0.5 M triethyl ammonium acetate, pH 6.0, and 10 ml. fractions of the column eluate were collected, until all the UV absorbing material in the column was eluted, usually about 1200 ml. of buffer being required. Tubes containing UV absorbing material pooled as necessary, and freeze-dried several times to remove

as much solvent as possible.

The various UV absorbing peaks obtained from the column were subjected to a further fractionation by paper electrophoresis on Whatman No. 1, or Whatman 3 MM paper in Buffer A. Separation of the various substances in each peak was carried on Locarte High Voltage Electrophoresis Equipment (Locarte Co., 24 Emperors Gate, London) at 80-100 mA for 4-5 hr. This also served to desalt the material and remove any residual traces of solvent. Guide strips were cut and stained for phosphorus with the spray reagent of Hanes and Isherwood (1949), phosphorus containing areas being eluted with distilled water, and freeze-dried to small volume.

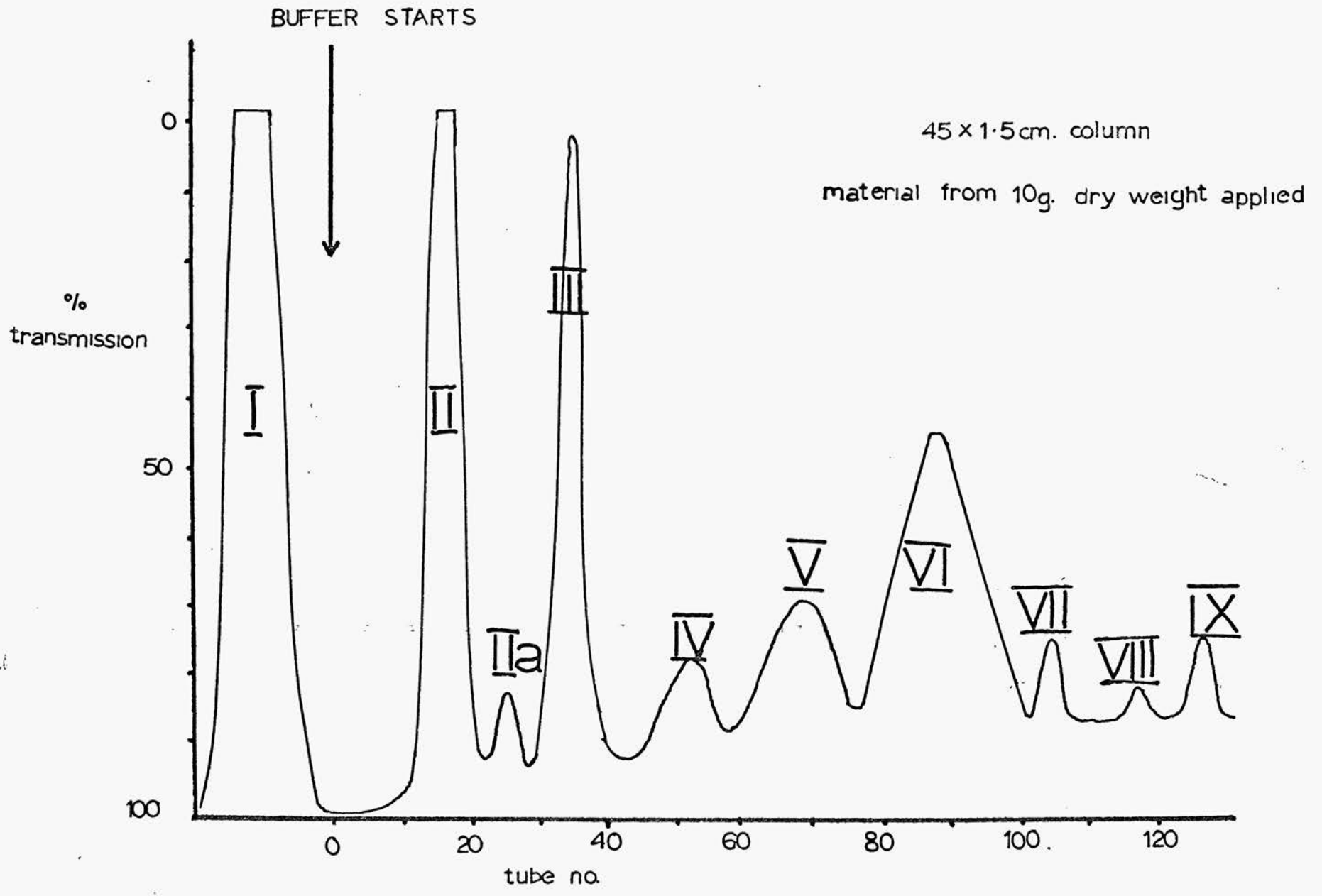
The fractions obtained by electrophoresis were subjected to ascending chromatography on thin layer plates of 0.3 mm. thickness prepared from MN 300 cellulose powder (Macherey, Nagel and Co., Duren, Germany) incorporating 2% (w/w) luminescent material (Type H 913, Green, Levy West Laboratories, Ltd., Harlow, England) in Solvents D and E.

Nucleotides, nucleosides, nucleobases, were visualised under illumination of 2537 Å (Hanovia Lamps, Slough, England) as dark blue spots on a bright green background. Less than 1 µg. could be detected in this way.

S53 Exponential-phase cells

Material from 5 g. dry weight of cells was applied to a 25 x 1 cm. column of ECTEOLA cellulose, material from 5-20 g.

FIGURE 26 S53 EXPONENTIAL-PHASE CELLS



dry weight to a 45 x 1.5 cm. column. A typical UV trace of the eluate from such a column is shown in Figure 26.

Peak I eluted from the column with distilled water contained a large amount of ultra-violet absorbing material, some 80% of the material applied to the top of the column, estimated by the optical density at 260 mu. The UV-absorbing material did not move from the origin on electrophoresis in Buffer A at 80-100 mA for 4 hr., and did not stain for phosphorus. Chromatographic analysis in Solvents D and E revealed the bulk of the material to be a mixture of nucleosides and nucleobases. All of the five commonly occurring purines and pyrmidines were detected, along with their nucleoside derivatives. Chromatographic analysis of Peak I in Solvents A and B did not reveal any material which stained with alkaline $AgNO_3$. Hydrolysis of Peak I in 0.01 N HCl for 10 min., followed by chromatography did not reveal any sugar component, nor were any released on hydrolysis with 1.0 N H_2SO_4 for 1 hr.

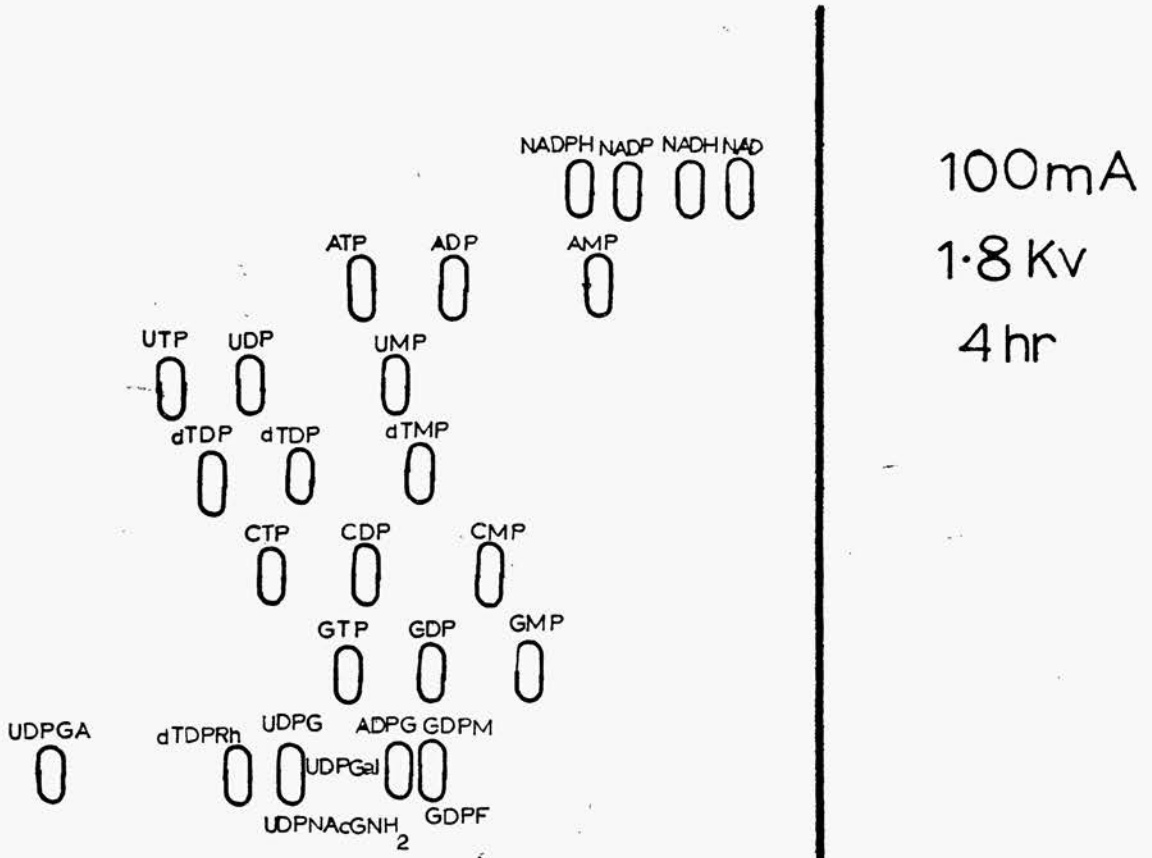
Peak II was composed largely of material which had the electrophoretic and chromatographic mobilities of NAD, NADH, and NADP. NADH was particularly easily visualised due to its additional fluorescence at 340 mu. Also present in Peak II was a small amount of material which moved towards the cathode on electrophoresis in Buffer A, staining with alkaline $AgNO_3$. On further examination this material appeared to have electro-

phoretic and chromatographic mobility in Buffer A, and Solvents A and B, similar to a sample of authentic KDO. Identification was further confirmed by performing a thiobarbituric colour reaction on both and examining the colour spectra, when both proved to have E_{max} of 550 mu. Also present in Peak II were small amounts of material with the chromatographic mobilities of CMP and UMP in Solvent E. Presumably KDO is present originally as CMPKDO, but being extremely labile, breaks down to give free KDO, which being acid is absorbed to the cellulose column, and the presence of CMP in the same peak may be significant. No other sugar components were detected in this peak, before or after hydrolysis with 0.01 N HCl and 1.0 N H_2SO_4.

Peak IIa appeared to be composed of small amounts of NAD, NADH, and NADP, but the bulk of the UV absorbing material moved in Solvents D and E with the chromatographic mobility of NADPH. The material also fluoresced strongly, similar to NADH. No sugar components were detected.

Peak III on hydrolysis with 0.01 N HCl at 100^{o} for 10 min. gave a mixture of sugar components which were identified by chromatography on thin-layer cellulose in Solvents A, B and C, as glucose, galactose, rhamnose, and fucose. Chromatography of material obtained by electrophoresis in Buffer A, revealed three main components which appeared to be homogeneous in Solvents D and E. In Solvent D they had the chromatographic

FIGURE 27 ELECTROPHORESIS OF NUCLEOTIDES IN BUFFER A



mobilities of UDP, dTDP, and GDP derivatives respectively. On hydrolysis of the compounds with 0.01 N HCl at 100° for 10 min., followed by chromatography in Solvents A and B, staining for sugars released with alkaline $AgNO_3$, and chromatography in Solvents D and E in order to detect nucleoside diphosphates released, the compounds were identified as dTDPRhamnose, GDPFucose, and a mixture of UDPG and UDPGal. dTDPRh and GDPF could be easily separated from each other and from UDPG and UDPGal by electrophoresis in Buffer A at 80-100 mA for 4-5 hr., but the systems available did not separate UDPG from UDPGal (Figure 27). In Solvents D and E, and on electrophoresis in Buffer A, UDPG and UDPGal moved as a single spot. However, the two compounds could be identified by hydrolysis in 0.01 N HCl at 100° for 10 min., followed by rechromatography in Solvents A, B, D, and E. In Solvents A and B, two sugar components having the mobilities of glucose and galactose were detected, but in Solvents D and E, only one component with the mobility of UDP was detected. The nucleotide sugars made up the bulk of the material in Peak III although there were other components present, among which were identified dTDP and GDP, perhaps a reflection of breakdown of the sugar components.

Peaks IV and V did not release any sugar components after hydrolysis in 0.01 N HCl at 100° for 10 min., and appeared to consist of a mixture of nucleoside di- and triphosphates. Material with the chromatographic mobilities in Solvents D and

E of ADP, ATP, UDP, and UTP were detected. However, hydrolysis of Peak V in 1.0 N H_2SO_4 at 100° for 1 hr. released sugars which had the chromatographic mobilities of glucose and mannose in Solvents A and B. Electrophoresis of Peak V in Buffer A revealed a single spot staining with alkaline $AgNO_3$ and for phosphorus with the electrophoretic mobility of G-6-P. Since this electrophoretic system does not distinguish different sugar monophosphates, it is concluded that the spot represents a mixture of glucose and mannose phosphates.

Peak VI has a distinct yellow colouration and is a useful marker peak. This yellow material, which also absorbs strongly in the UV, appears to make up the bulk of the material in this peak, and it moves slowly towards the cathode on electrophoresis in Buffer A. It is concluded that this material is either flavine mononucleotide (FMN) or flavinadenine dinucleotide (FAD), probably the latter since hydrolysis of the material in 0.01 N HCl at 100° for 10 min. released material with the chromatographic mobilities of AMP and ADP. Peak VI after hydrolysis with 0.01 N HCl gives a positive colour reaction with Morgan-Elson reagent for N-acetyl amino sugars. Material isolated by electrophoresis in Buffer A had the same chromatographic mobility in Solvents D and E as authentic $UDPNAcGNH_2$, and on hydrolysis with 0.01 N HCl at 100° for 10 min. yielded material with the chromatographic mobility of UDP in Solvents D and E, and material with the chromatographic mobility of $NAcGNH_2$ in

Solvents A and B. However, electrophoresis of the hydrolysed compound in Buffer A revealed that the sugar component was not homogeneous, two spots staining with alkaline $AgNO_3$ being observed, one remaining at the origin which is presumed to be $NAcGNH_2$, and the other moving towards the cathode. This particular spot had the electrophoretic mobility similar to that of a sample of N-acetyl muramic acid which was prepared by acetylating muramic acid. N-acetyl muramic acid could not be distinguished from N-acetyl glucosamine in Solvents A, B and C, so its presence was never unequivocally confirmed, but it is concluded that Peak VI contains $UDPNAcGNH_2$ and possibly UDPNAc MurA. Other material is present in the peak in small amounts but was not identified further. No further sugars were detected.

Peak VII is electrophoretically and chromatographically homogeneous, and has the mobility of authentic UDPGA. Identification was confirmed by hydrolysis and chromatography of the nucleoside diphosphate and sugar released, in Solvents A, B, D and E.

Peak VIII streaks in electrophoresis and chromatography and does not release any sugar on hydrolysis in either 0.01 N HCl or 1.0 N H_2SO_4. It fluoresces strongly and may be small molecular weight RNA or oligonucleotides.

Peak IX resembles Peak VIII and the conclusions are similar.

No other material was eluted from the column with 0.5 M buffer. Further elution with 1.0 M buffer pH 4.0 produced a small amount of material similar in behaviour to Peaks VIII and IX.

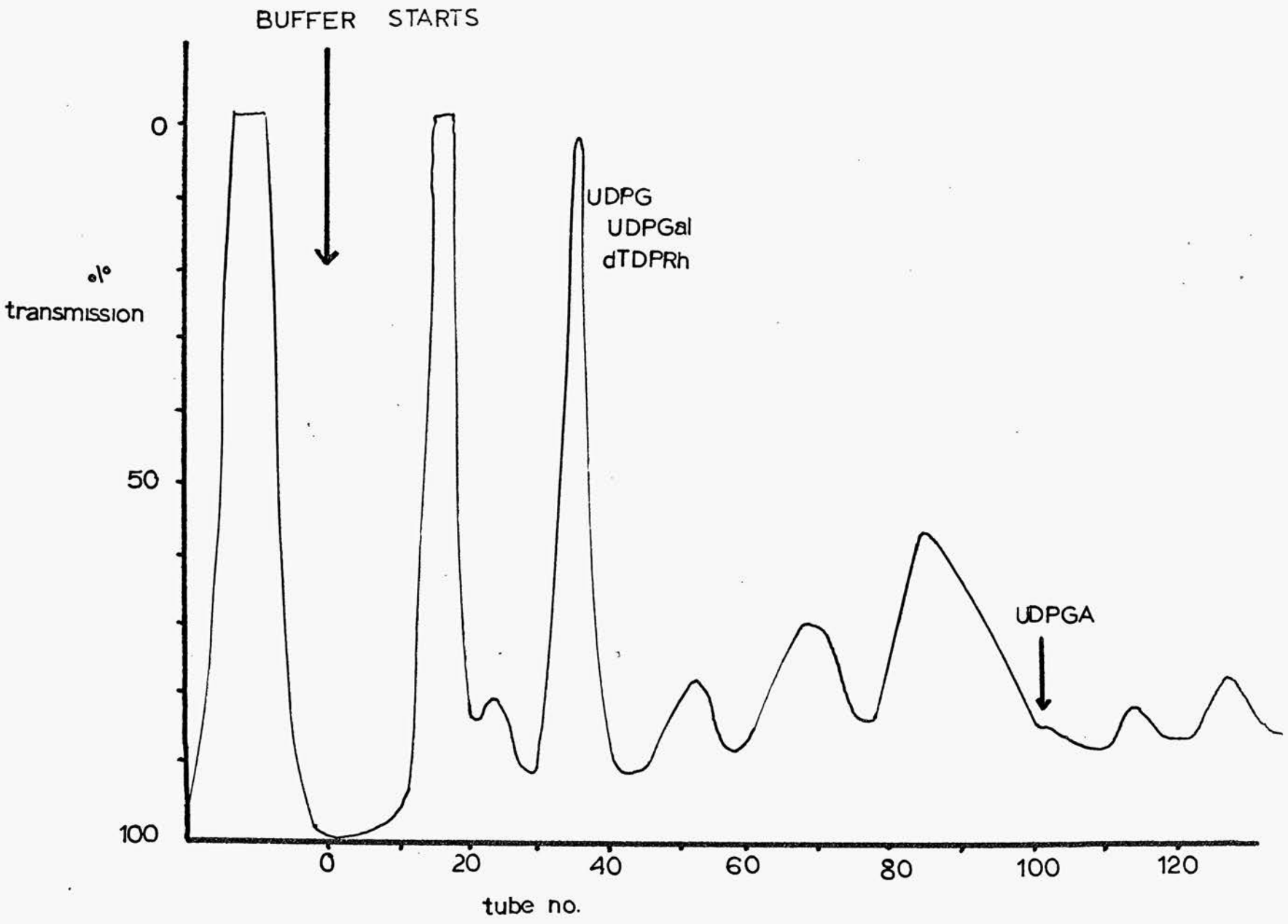
S53 Stationary-phase cells

The overall yield of UV absorbing material calculated as optical density units at 260 mμ was substantially the same as that obtained from exponential-phase cells. The only difference apparent from a comparison of the soluble pool with that of exponential-phase cells is that the size of the peak containing the yellow material is considerably reduced. The yellow material has a maximum absorption at 450 mμ and on the basis of this there appeared to be about 50% of the yellow material found in exponential-phase cells, presumably a reflection of the reduction in oxidative metabolism of the stationary-phase cells. The nucleotide sugars to be found are exactly the same as in the exponential-phase cells.

S61 Stationary-phase and exponential-phase cells

In view of the obvious physical differences in polysaccharide between S53 and S61, the nucleotide pool of both exponential and stationary phase cells was examined to determine any differences in the nucleotide sugar pattern. However, no

FIGURE 28 S56 STATIONARY-PHASE CELLS



qualitative differences were detected between S53 and S61, both in the stationary-phase and exponential-phase cells, the analysis being identical to the pattern outlined for S53, as far as could be determined.

S22, S22-23, S8, S53c

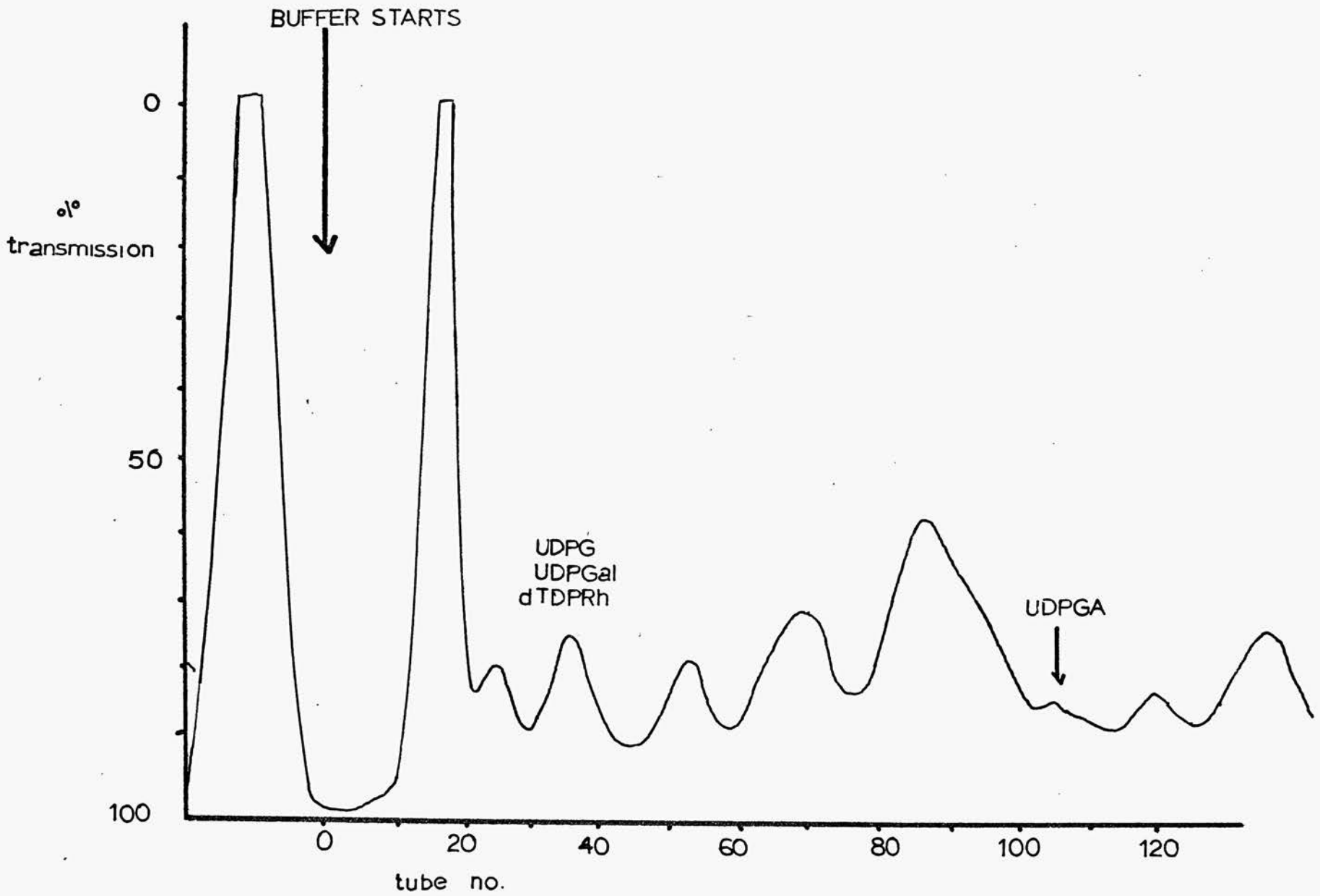
Stationary-phase cells were examined in all cases, and again the pattern observed was identical to that of S53 as far as could be determined.

S56, S22M, CA1OR, S5

All of these strains do not produce Colanic acid on ordinary media, or at least only very little after long incubation at low temperature. Stationary-phase cells of these strains were examined and examination of the nucleotide pools revealed a similar pattern outlined by the example illustrated in Figure 28. In all cases the only obvious difference in the trace compared with one from S53 stationary-phase cells, is that the size of the UDPGA peak is much smaller. Qualitative analysis in all cases revealed that UDPG, UDPGal, dTDPRh, were all present. UDPGA was also present but clearly in only small amount. The most important difference was that GDPF was not detected, nor was any other fucose derivative.

S23 This strain did not produce Colanic acid under all conditions tested, and analysis of the nucleotide pool revealed

FIGURE 29 CA 10 STATIONARY - PHASE CELLS



the pattern to be substantially the same as that of the S56, S22M, CA1OR, S5 group, with the exception that GDPF was detected.

CA3 This non-mucoid strain is defective in the enzyme UDPG-4-Epimerase, and it might be expected to lack the nucleotide sugar UDPGal. An examination of the peak containing the bulk of the nucleotide sugar material in the cell revealed only UDPG and dTDPRh. Qualitative analysis appeared to indicate a small amount of UDPGA, a pattern resembling the S22M, CA1OR group, with the exception of the deletion of UDPGal. No derivatives of fucose were detected. It might have been expected that dTDPRh would accumulate since the LPS of the strain is defective, and the site of attachment for rhamnose missing, but this does not appear to be the case.

CA1O This strain is deficient in UDPG pyrophosphorylase and might be expected to lack the nucleotide sugar UDPG and in addition UDPGal and UDPGA since both are derived from UDPG. A typical UV trace from column chromatography of the nucleotide pool is outlined in Figure 29. As can be seen the peak normally associated with UDPG and UDPGal has practically disappeared, as has the peak associated with UDPGA. Qualitative analysis revealed that contrary to what was expected very small amounts of UDPG, UDPGal and UDPGA were detected, suggesting that the enzyme defect is leaky, or more likely that revertants arise in the culture similar to CA1OR, and these are responsible for the UDPG, UDPGal and UDPGA. It was also noticed that only

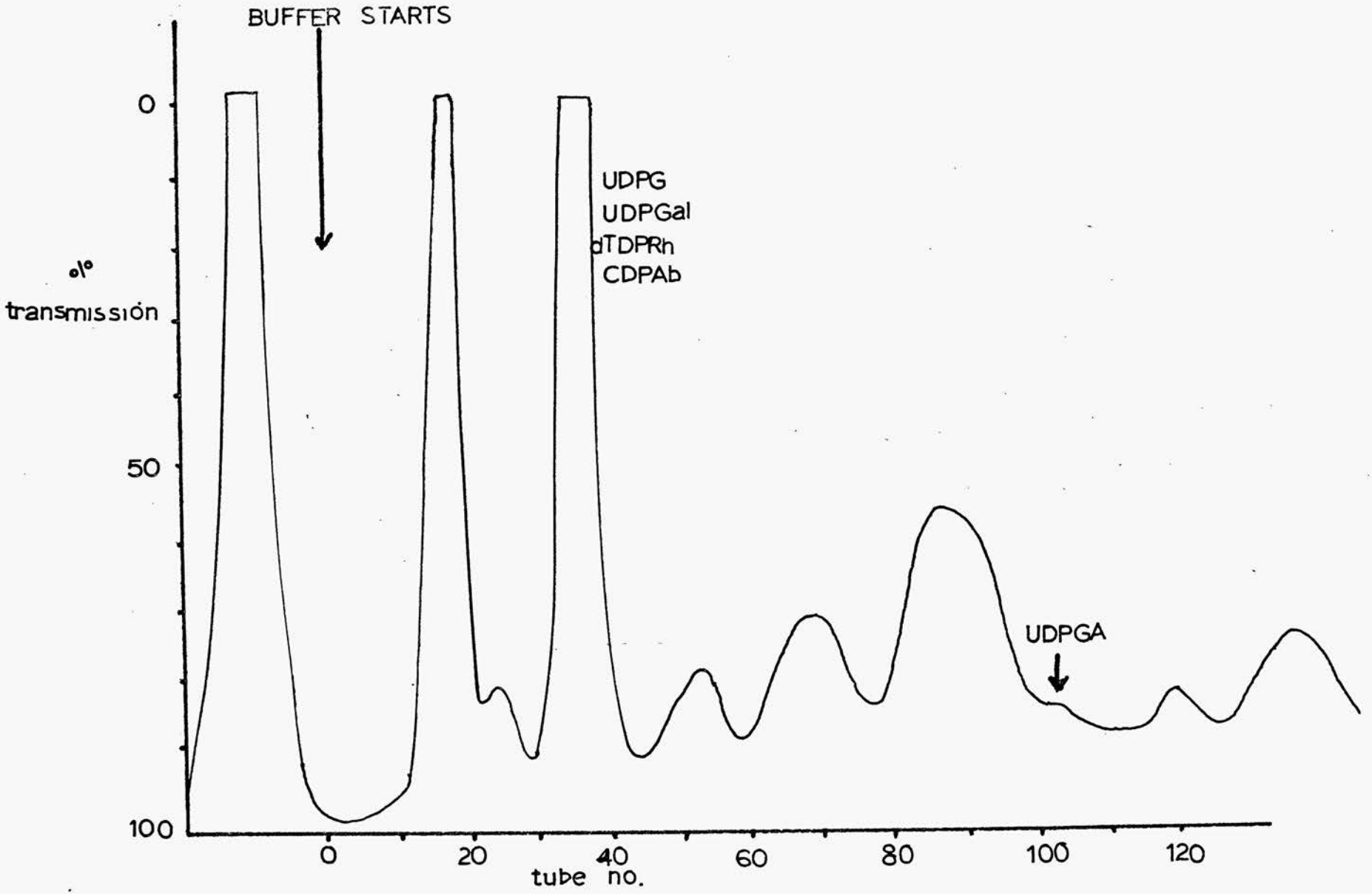
very small amounts of dTDPRh were present, far less being present than in CA3 for example, yet both strains possess deficient LPS without a site of attachment for rhamnose, and one would expect the levels of dTDPRh in each case to be substantially the same. It may be that in the absence of UDPG, dTDPG is used for some reactions resulting in very little dTDPG being available for the synthesis of dTDPRh, or it may be that the defect in UDPG pyrophosphorylase also causes a defect in dTDPG pyrophosphorylase as described by Wu (1965) resulting in the absence of dTDPRh.

S53/1/2/3 All three strains appeared to be identical, and examination of the nucleotide pool revealed no apparent differences, both qualitative or quantitative from that of S53. GDPF, dTDPRh, UDPG, UDPGal, and UDPGA all were present.

Aerobacter cloacae 5920, Salmonella typhimurium 1098 Both strains are highly mucoid on all types of media and analysis of the nucleotide pool of both revealed the pattern to be substantially the same as that of S53 and other mucoid K12 strains. The nucleotide sugars UDPG, UDPGal, dTDPRh, UDPGA, and GDPF were detected in both strains. As far as could be detected there was no nucleotide derivative of the dideoxyhexose present in the S. typhimurium LPS. The only qualitative differences between the mucoid K12 strains and these two strains was that two yellow peaks were observed to be eluted from the ECTEOLA

FIGURE 30

S. typhimurium SL 1543
STATIONARY-PHASE CELLS



columns. One appeared to be the same as that observed in column chromatography of nucleotides from K12 derivatives in that it appeared at the same point and the peak also contained $UDPNAcGNH_2$, but the other appeared earlier in the region normally associated with sugar phosphates. This particular substance moved slower in electrophoresis in Buffer A than the other, and it may be another flavine derivative such as FMN.

S. typhimurium 1542 and 1543 These strains were of particular interest because 1542 has been said to have a defect in the biosynthesis of GDPM (Nikaido et al., 1967) and the LPS contains only the core sugars glucose, galactose, and N-acetyl glucosamine, whereas the wild-type contains in addition mannose, rhamnose and abequose. 1543 was detected as a mucoid variant of 1542 by these workers and the exopolysaccharide produced contains galactose, glucuronic acid, glucose, and fucose, and is sensitive to depolymerase enzymes, indicating that it is Colanic acid. The strain only produces very small amounts of Colanic acid on long incubation at low temperature, but the significant point is the possession of this fucose-containing exopolysaccharide, since it might be expected that the strain would be unable to synthesise GDPF because of its inability to synthesise GDPM.

Column chromatography of the nucleotide pools of both strains appeared to suggest that they were substantially the same, and the UV trace of the eluate from a column analysis of the nucleotides of 1543 is illustrated in Figure 30. The most

striking feature is the size of the peak in the UDPG-UDPGal region. Examination revealed UDPG, UDPGal, and large amounts of dTDPRh and a CDP sugar which is probably abequose. It seems likely that this strain accumulates the nucleotide derivatives of the O-specific sugars. However, no fucose derivative was detected in either strain, despite large amounts of starting material. Both nucleotide pools contained small amounts of UDPGA.

NUCLEOTIDE POOLS OF STRAINS GROWN IN PFA CONTAINING MEDIUM

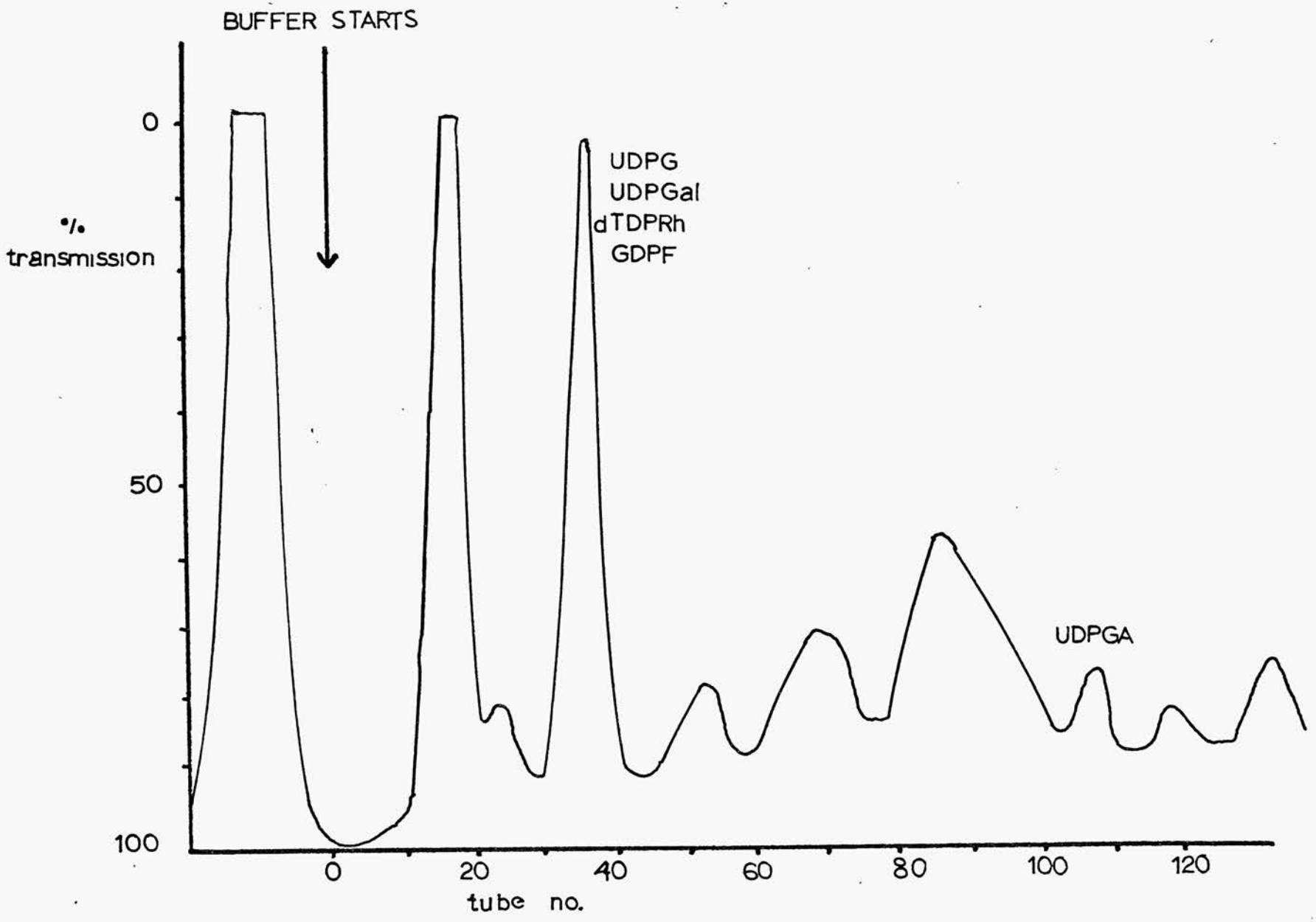
Since many strains which do not produce Colanic acid on ordinary media can be induced to produce Colanic acid grown in the presence of p-fluorophenylalanine (PFA), several of such strains were grown on PFA media and their nucleotide pools examined. The strains S53/1/2/3, S23, CA3 and CA10 were also of interest since they are non-mucoid even in the presence of PFA.

Strains were grown in liquid Minimal A PFA or liquid Minimal B PFA medium depending on growth requirements, for 7 days at 25°. Generally very low yields of cells were obtained of the order of 0.2 g. dry weight/l.

S56, S22M, CA10R, S5

All showed essentially the same pattern, and a typical UV trace of the column analysis of one of the nucleotide pools,

FIGURE 31 S 56 STATIONARY-PHASE CELLS GROWN IN THE PRESENCE OF PFA



that of S56, is shown in Figure 31. It can be seen that, compared with the UV trace from cells grown in nutrient broth, the size of the UDPGA peak is considerably larger. Analysis of the nucleotide sugars present revealed UDPG, UDPGal, UDPGA, and GDPF, the presence of the last mentioned having failed to be detected in cells grown in nutrient broth.

CA3, CA10

In both cases the qualitative composition of the nucleotide pool remained similar to that obtained when the cells were grown in nutrient broth, with the exception that GDPF was detectable in both strains. The size of the UDPGA peak was considerably increased in both instances.

S23, S53/1, S53/2, S53/3

No change was observed from the composition of the nucleotide pool when the cells were grown in nutrient broth.

QUANTITATIVE ANALYSIS OF NUCLEOTIDE SUGARS

In view of the apparent differences in the quantities of certain nucleotide sugars present in various strains, in particular the difference in level of UDPGA between mucoid and non-mucoid strains, several of the nucleotide sugars present were assayed by various methods.

UDPGA was determined on material eluted from ECTEOLA

TABLE 14: NUCLEOTIDE SUGAR LEVELS OF VARIOUS STRAINS

Organism	Mucoid		UDPG		UDPGal		UDPGA		TDPRh		GDPF	
	NB	PFA	NB	PFA	NB	PFA	NB	PFA	NB	PFA	NB	PFA
S53 Exponential	++	++	0.095	0	0.068	0	1.29	0	0.81	0	0.05	0
S53 Stationary	++	++	0.148	0	0.082	0	1.63	0	0.87	0	0.05	0
S61 Exponential	++	++	0.137	0	0.081	0	1.39	0	0	0	0.059	0
S61 Stationary	++	++	0.161	0	0.072	0	1.48	0	0	0	0.072	0
S53/1	-	-	0.120	0.113	0.041	0.038	0.71	0.81	0	0	0.039	0.029
S53/2	-	-	0.097	0.083	0.057	0.071	0.64	0.69	0	0	0.041	0.071
S53/3	-	-	0.122	0.097	0.061	0.068	0.59	0.47	0	0	0.037	0.059
S22	++	++	0.097	0	0.068	0	1.15	0	0	0	0.037	0
S23	-	-	0.085	0.11	0.049	0.052	0.11	0.14	0	0	0.049	0.037
S22-23	++	++	0.150	0	0.073	0	0.950	0	0	0	0.039	0
S 8	++	++	0.097	0	0.068	0	1.01	0	0	0	0.052	0
S56	-	+	0.095	0.087	0.062	0.081	0.029	0.47	0	0	-	0.071
S22M	-	++	0.110	0.072	0.097	0.078	0.052	0.68	0	0	-	0.059
CA1OR	-	++	0.083	0.059	0.091	0.062	0.041	0.80	0	0	-	0.041
CA1O	-	-	0.011	0.023	0.011	0.032	0.010	0.047	0	0	-	0.037
CA 3	-	-	0.083	0.091	-	-	0.062	0.31	0	0	-	0.029

Organism	Mucoid		UDPG		UDPGal		UDPGA		TDPRh		GDPF	
	NB	PFA	NB	PFA	NB	PFA	NB	PFA	NB	PFA	NB	PFA
A. cloacae 5920	++	++	0.130	0	0.097	0	1.80	0	0	0	0.027	0
S. typhimurium 1098	++	++	0.158	0	0.082	0	1.10	0	0	0	0.062	0
S. typhimurium 1542	-	-	0.137	0	0.081	0	0.052	0	1.47	0	-	0
S. typhimurium 1543	±	±	0.119	0	0.064	0	0.068	0	1.81	0	-	0

Cells are in stationary phase unless otherwise indicated.

NB indicates nutrient broth or nutrient agar; PFA indicates solid or liquid medium containing PFA.

Results expressed as μM sugar nucleotide/gm. dry wt. of cells.

0 not tried.

- not detectable.

cellulose columns by the carbazole method of Bowness (1957). GDPF and dTDPRh were determined on material eluted from columns, after a further purification by electrophoresis in Buffer A at 80-100 mA for 4-5 hr., using the cysteine-H_2SO_4 method of Dische and Shettles (1951). UDPGal was determined on material eluted from columns, after hydrolysis of the material in 0.01 N HCl at 100° for 10 min., the galactose released being determined using the Galactostat reagent (Worthington Biochemical Corporation, New Jersey). UDPG was determined on material eluted from columns with an enzyme system involving UDPG dehydrogenase in a modification of the method described by Leloir and Paladini (1951). An assay mixture consisted of: 60 µl. 1.0 M glycine-NaOH pH 8.7; 1 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M NAD; 500 µl. H_2O; 100 µl. UDPG dehydrogenase (500 units); 10-100 µl. sample. Incubation at 30° for 15 min. was carried out, and the absorption at 340 mu measured.

The results are outlined in Table 14 which illustrates the nucleotide sugar levels in various strains, both mucoid and non-mucoid on all types of media, and those mucoid only when grown in the presence of PFA.

It can be seen that those sugar nucleotides present in highest concentration are UDPGA and dTDPRh, and that in the two cases examined the level of sugar nucleotides in stationary-phase cells seems marginally higher than in exponential-phase cells. With the exception of the mutant strains CA3 and CA10, all the strains have roughly the same level of UDPG and UDPGal,

although in the strains which produce exopolysaccharide on all types of media, the level of UDPGal is marginally higher. The striking differences evident in the comparison of a mucoid strain such as S53 with a strain such as CA1OR which is mucoid only on PFA, are that grown on nutrient broth the mucoid strains have some 10-20 times the level of UDPGA found in the others, and that GDPF can be detected in mucoid strains only, with several exceptions which will be discussed. The strains such as CA1OR when grown on PFA produce exopolysaccharide, and under these conditions the levels of UDPGA reach levels comparable to those of strains such as S53, and GDPF becomes detectable in the nucleotide pool.

The exceptions to the general pattern are the strains S53/1, S53/2, S53/3 and S23. The first three are probably identical, and in all cases the levels of UDPG, UDPGal, GDPF and UDPGA were comparable with S53 although the UDPGA level is reduced by about 50%. However, the strains do not produce exopoly-saccharide, even on PFA media, and are thus clearly different from any of the other strains. The strain S23 is also unusual in that GDPF can be detected at almost the same level as found in mucoid strains, but UDPGA is present in low level only. These strains are not mucoid on PFA medium and the levels of UDPG, UDPGal, GDPF and UDPGA do not change on growing the cells in the presence of PFA.

Salmonella typhimurium 1542 and 1543 were examined to determine if any fucose derivative was present, particularly

since 1543 produces small amounts of Colanic acid on long incubation. Both strains accumulate large amounts of dTDPRh and CDP dideoxyhexose (probably abequose), but no fucose derivative was detected in either. Both had low levels of UDPGA comparable with strains like CA1OR, and others which were non-mucoid on ordinary media.

ATTEMPTS TO ISOLATE AND IDENTIFY A NUCLEOTIDE DERIVATIVE OF THE UNKNOWN SUGAR IN COLANIC ACID

(a) A sample of the unknown sugar said to be present in Colanic acid was provided by Dr C.W. McCleary. On thin-layer in Solvents A and B, it had a mobility similar to the lactone of glucuronic acid, which is considerably faster moving than fucose or rhamnose. In all the investigations of nucleotide pools in mucoid and non-mucoid strains, no derivative of a sugar with such a chromatographic mobility was observed. It was thought that the sugar might be destroyed by the extraction procedures, or by hydrolysis in 0.01 N HCl, but an authentic sample of the sugar survived such procedures without appreciable destruction.
(b) Experiments with washed cell suspensions of S53 had shown that suspended in a suitable medium with glucose as a carbon and energy source, exopolysaccharide synthesis commences after 2-3 min., increased to a maximum rate after 30-40 min., and continued at a constant level for up to five hours in conditions of carbon excess.

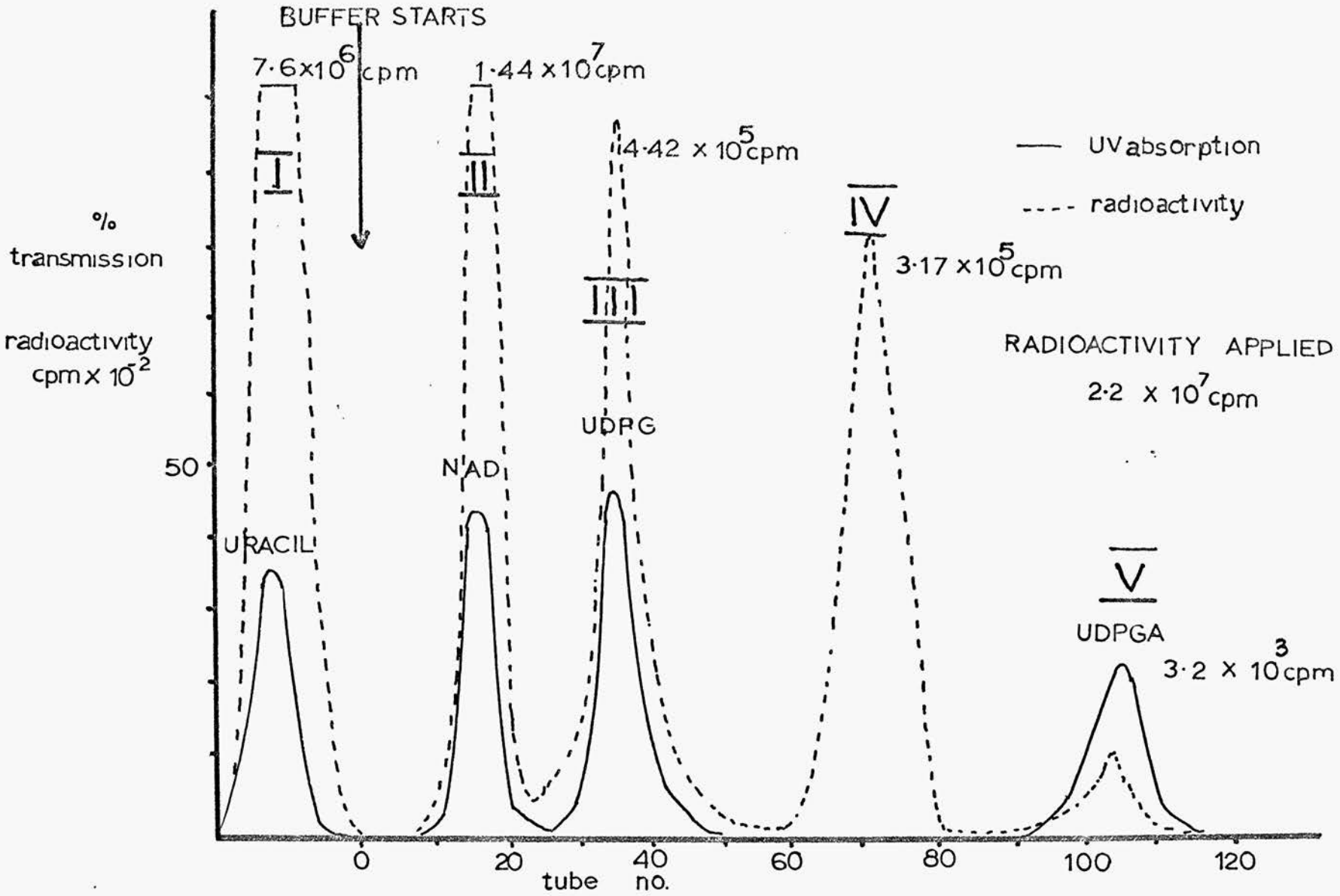
Using ^{14}C-glucose an attempt was made to label the sugar nucleotides of S53, in order to detect any small amount of the unknown derivative that might be present.

S53 cells were grown overnight in shake culture in YE medium at 30°, and centrifuged at 50,000 g to try and remove them from the exopolysaccharide as much as possible. The cells were washed several times with saline to remove residual traces of exopolysaccharide, and cells from 1 l. of culture were uniformly resuspended in 200 ml. of a medium which was identical to YE medium, but devoid of any nitrogen source. Incubation was carried out for 1 hr. at 30° with gentle shaking, and after this time 20 ml. 10% (w/v) glucose was added, along with 20 uC. uniformly labelled ^{14}C-glucose. Incubation was continued for a further 2 hr., the cells spun down at 50,000 g, washed at 0°, the supernate and washings precipitated in acetone and deproteinised, and the cells subjected to ethanol extraction, followed by LPS extraction and purification.

It was considered that under these conditions of nitrogen deprivation, it was unlikely that ^{14}C-glucose would be incorporated into anything other than exopolysaccharide, and in addition the stationary phase of the cells is unlikely to be an active LPS synthesising period.

In such experiments this hypothesis appeared to be borne out since under these conditions after 2 hr. incubation less than 0.1% of the total radioactivity added was incorporated

FIGURE 32 CHROMATOGRAPHY OF S53 ^{14}C-LABELLED SOLUBLE POOL ON ECTEOLA CELLULOSE



into LPS, and generally 8-12% of the total activity added was incorporated into exopolysaccharide, and some 9-11% into the ethanol extractable material, while some 15-20% was blown off as CO_2.

The ethanol extractable material was chromatographed on ECTEOLA cellulose columns, NAD, UDPG, UDPGA, and uracil, being added as marker substances. The effluent from the column was continuously monitored at 254 mμ and aliquots from the fractions collected were counted for radioactivity. The result of such a procedure is illustrated in Figure 32. As can be seen from the figure 2.2×10^7 cpm applied to the column, some 35.4% was eluted in the uracil region with distilled water (Peak I), 65.2% was eluted in the NAD region (Peak II), 0.19% in the UDPG region (Peak III), 0.14% in a region between UDPG and UDPGal (Peak IV) and 0.0014% in the UDPGA region (Peak V), representing a 99.83% recovery of radioactivity.

Peak I was subjected to electrophoresis in Buffer A at 80-100 mA for 4 hr., when all of the radioactivity remained at the origin. Chromatography of the peak on thin layer in Solvents A and B revealed that most of the radioactivity migrated with the mobility of glucose, indicating that a considerable amount of the original medium had not been washed from the cells adequately. The only other radioactive area on the chromatogram was a small area extending from the origin and streaking a small distance up the chromatogram. On chromatography in Solvents A and B

before and after hydrolysis in 0.01 N HCl at 100° for 10 min., radioactivity was looked for in the area where the unknown sugar would be expected to run, but none was detected.

Peak II on electrophoresis in Buffer A at 80-100 mA for 4 hr., radioactive material streaked from the origin as far as the region in which sugar monophosphates run. On thin-layer in Solvent D, all of the radioactivity ran with or near the solvent front. In Solvents A and B before and after hydrolysis with 0.01 N HCl at 100° for 10 min. the radioactive material streaked from the origin over a short distance to the region where glucosamine runs. No radioactivity could be detected in the area where the unknown sugar was expected to run.

Peak III on electrophoresis in Buffer A at 80-100 mA for 4 hr. showed three radioactive regions. Comparison with standard nucleotides UDPG, GDPM, and a sample of dTDPRh which was obtained from the nucleotide pool of S. typhimurium 1543 by electrophoresis and TLC, indicated that the three regions corresponded to these three nucleotide sugars. Further confirmation was obtained by chromatography on thin layer in Solvent D, where the radioactivity again ran in three regions, corresponding to UDP, GDP, and dTDP derivatives. On elution of these regions and hydrolysis in 0.01 N HCl at 100° for 10 min., all of the radioactivity from the UDP region ran in Solvent B in the glucose-galactose region, that from the GDP

region in the fucose region, and that from the dTDP region in the rhamnose region. Again radioactivity was looked for in the area where the unknown sugar might be expected to run, but none was found.

Peak IV on electrophoresis in Buffer A appeared to be radioactively homogeneous, and the radioactivity migrated with the mobility of a sugar phosphate. Hydrolysis in 0.01 N HCl at 100° for 10 min. followed by chromatography in Solvent B gave a radioactive spot which moved only a small way from the origin, again similar to a sugar phosphate, but hydrolysis in 1.0 N H_2SO_4 at 100° for 30 min. released all the radioactivity which ran in Solvent B in the glucose-mannose region. No radioactivity was detected in Solvent B in the area where the unknown sugar was expected.

Peak V was electrophoretically and chromatographically homogeneous. In Solvents D and E the radioactivity had the mobility of authentic UDPGA. Hydrolysis in 0.01 N HCl at 100° for 10 min. released all the radioactivity which had the mobility of glucuronic acid in Solvents A and B. No other radioactivity was detected.

Precisely what the material is in Peaks I and II, which makes up the vast majority of the radioactivity in the soluble pool, is not known, although it seems likely from its chromato-

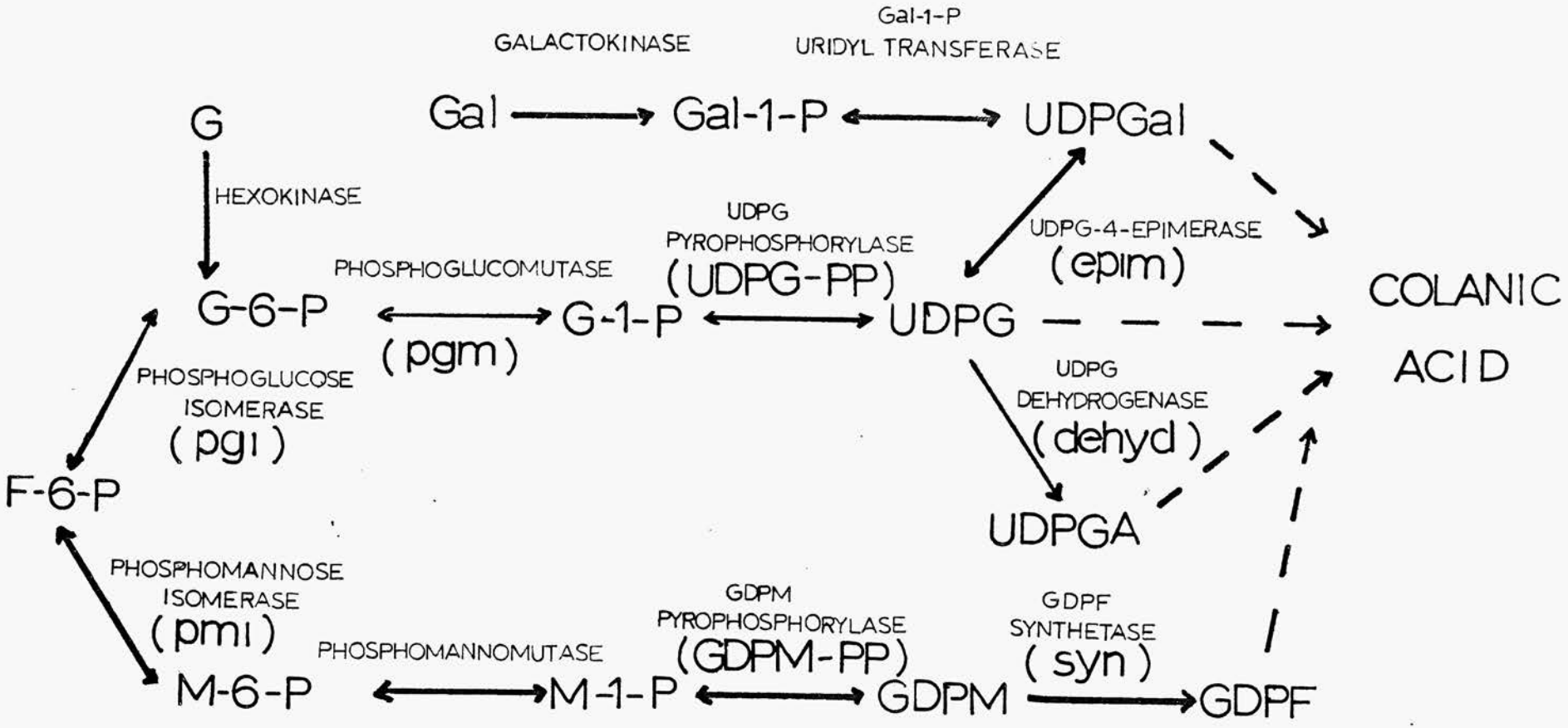
graphic and electrophoretic behaviour, and the conditions of ^{14}C-glucose incorporation, that it is made up of a mixture of small molecular weight material, charged and uncharged, from the intermediary energy metabolism of the cell such as the TCA cycle.

(c) Since the structure of the unknown sugar proposed by McCleary (Figure 24) has a C methyl group, it was considered that this group might be introduced by a reaction involving the CH_3 group of methionine, since methionine is known to participate in transmethylation reactions (Bray and Shemin, 1958).

S53 cells were grown overnight in shake culture in Minimal A medium supplemented with 0.002% (w/v) methionine, at 30°. The cells were removed by centrifugation at 20,000 g, washed twice with saline, and cells from 1 l. resuspended in 200 ml. of the same medium. Incubation at 30° with gentle shaking was carried out for 1 hr., when 10 μC $^{14}CH_3$-methionine was added. After a further 2 hr. incubation, the cells were centrifuged at 0° at 20,000 g, and washed several times with saline until the radioactivity of the washings was reduced to near background level. The combined supernate and washings were subjected to acetone precipitation and exopolysaccharide purification procedures, and the cells were extracted with boiling ethanol.

Under these conditions the exopolysaccharide produced did not become labelled, but methionine appeared to be taken up by the cells since the initial acetone precipitate was radioactive,

FIGURE 33 POSTULATED PATHWAY INVOLVED IN THE BIOSYNTHESIS OF COLANIC ACID



but gradually lost its radioactivity during deproteinisation procedures. Chromatography of the soluble pool on ECTEOLA cellulose as previously described revealed two radioactive peaks in the eluate from the column, one in the nucleoside-nucleobase region, the other in the NAD region. Both peaks on chromatography on thin-layer cellulose in water saturated phenol with a trace of NH_3, gave a single radioactive spot with the same chromatographic mobility of methionine. The difference between the two peaks may be a reflection of the Zwitterion properties of the molecule.

THE BIOSYNTHETIC PATHWAY OF COLANIC ACID

In the light of the results obtained on analysis of the nucleotide pools of mucoid and non-mucoid strains, it seemed likely that the pathway operative in Colanic acid biosynthesis is as illustrated in Figure 33. The pathways leading to the synthesis of the sugar nucleotides have been shown in various organisms, including *E. coli*, but all of these reactions have not yet been shown in any one strain of *E. coli*.

Cell-free extracts were obtained by growing cells in shake culture in broth, centrifuging the cells at 20,000 *g* at 0°, washing the cells twice with ice-cold saline, suspending cells from 500 ml. culture in 10 ml. ice-cold distilled water, and disrupting the cells with ultrasonic disintegration for 90 sec. The cell debris was centrifuged at 40,000 *g* at 0° and the

supernate kept in crushed ice. Enzymic activity was retained for many months at -20°, but analyses were generally carried out with freshly prepared material.

Assays were performed using modifications of well-known techniques, wherever possible. Methods for detecting GDPF synthetase and assaying UDPG dehydrogenase and UDPG-4-Epimerase were developed from existing methods as described.

UDPG-4-Epimerase was assayed by a modification of the two-step procedure described by Imae, Morikawa and Kurahashi (1964), using 100 ul. cell-free extract in the first reaction mixture, in 75 x 1 mm. test-tubes.

Assay mixture: 30 µl. 1.0 M glycine-NaOH pH 8.7; 20 µl. 0.005 M UDPGal; 2 µl. 1.0 M $MgCl_2$; 2 µl. 0.02 M NAD; 100 µl. H_2O. Incubation at room temperature was carried out for 0, 1, 2, 3, 5, 10 min., the reaction being stopped at these times by immersing the tubes in a boiling water bath for 90 sec., then transferring them to crushed ice. The precipitated protein was centrifuged at 1,000 g, and the supernate assayed for UDPG by the method previously described.

GDPF synthetase was detected by a modification of the method described by Ginsburg (1960). In the method described, the assay depends on the NADPH dependent reduction of GDP-4-keto-D-rhamnose which is formed from GDPM in the system. However, it was found in the E. coli systems under study that so much non-specific oxidation of NADPH took place that this

method could not be used, so the chromatographic appearance of fucose was taken as an indication of the enzyme activity instead.

Assay mixture: 50 µl. 1.0 M Tris-HCl pH 8.0; 10 µl. 0.02 M NADPH; 10 µl. 0.1 M GDPM; 5 µl. 1.0 M $MgCl_2$; 300 µl. H_2O. 500 µl. cell-free extract was added and incubation carried out for 0, 15, 30 and 60 min. at 30°, the reaction being stopped at these times by heating in a boiling water bath for 90 sec., followed by transfer to crushed ice. The precipitated protein was centrifuged at 1,000 g, and the supernate acidified to pH 1.0 with HCl. Heating was carried out at 100° for 10 min., the mixture freeze-dried, and desalted by electrophoresis in Buffer A at 80-100 mA for 30 min. The area of the origin was eluted with distilled water, concentrated, and subjected to chromatography on thin-layer cellulose in Solvents A and B, to determine the appearance of fucose.

UDPG dehydrogenase was assayed by a modification of the method described by Leloir and Paladini (1951). The method described depends on the NAD dependent oxidation of UDPG, the appearance of NADH being followed spectrophotometrically. However, in the system under examination there was so much non-specific reduction of NAD that this detection system could not be used, and a system for the detection of UDPGA was devised.

Assay mixture: 100 µl. 1.0 M glycine-NaOH pH 8.7; 10 µl. 1.0 M $MgCl_2$; 20 µl. 0.02 M UDPG; 500 µl. H_2O; 1 µl. ^{14}C UDPG (0.25 µC).

It was found that the bulk of the enzymic activity appeared to be bound to particulate matter, so broken cell preparations were used, prepared in the same way as the cell-free extracts by ultrasonic disintegration, but omitting the centrifugation at 40,000 g. After the addition of 100 µl. broken cell preparation, incubation was carried out at 30° for 0, 10, 20 and 30 min., the reaction being stopped at these times by placing the tubes in a boiling water bath for 90 sec. followed by transfer to crushed ice. Precipitated protein was removed by centrifugation at 1,000 g and the supernate subjected to electrophoresis in Buffer A at 80-100 mA for 6 hr. The areas corresponding to UDPG and UDPGA were cut out and counted.

Of the assay methods used, the method for the assay of GDPM pyrophosphorylase is extremely poor, since the final detection step, an increase in absorption at 340 mµ, depends on no fewer than three enzymic reactions in sequence. It was found that there was a great deal of non-specific absorption at 340 mµ, and while in the case of mucoid organisms such as S53, there appeared to be a significant difference in absorption between the complete assay mixture and various controls, with other strains the difference was not significant. The method was used simply as a detection system for GDPM pyrophosphorylase, and not as a true assay system, and whereas a positive result is probably significant, a negative result is much less

so, since the margins of error with this technique are so great.

The GDPF synthetase detection method devised is also simply a detection method and not an assay method as it stands, but here the technique is sufficiently sensitive that positive and negative results are equally significant.

ENZYMES PRESENT IN S53

Extracts of a typical highly mucoid strain, S53, were examined for the various enzymes believed to be operative in the biosynthesis of Colanic acid. The enzymes phosphoglucose isomerase, phosphomannose isomerase, phosphoglucomutase, UDPG pyrophosphorylase, GDPM pyrophosphorylase, UDPG-4-Epimerase, and UDPG dehydrogenase were detected when the cells were grown in nutrient broth. Cell-free extracts would also catalyse the conversion of GDPM to GDPF, but not ADPM or UDPM. Grown in glucose broth, the cells also produced hexokinase, and grown in galactose broth the cells produced galactokinase and gal-1-Ⓟ uridyl transferase. The only enzyme not assayed was phospho-mannomutase.

ENZYME LEVELS

Kang and Markowitz (1965) have already demonstrated that strains of *E. coli* K12 which become mucoid when grown in the presence of PFA, produce elevated levels of certain enzymes supposedly concerned in exopolysaccharide synthesis, notably

TABLE 15: ENZYME LEVELS OF VARIOUS STRAINS

Organism	Mucoid on ordinary medium	Mucoid on PFA	PGI	PGM	UDPG PP	EPIM	DEHYD	PMI	GDPM PP	GDPF SYN
S53	++	++	13.3	5.1	0.92	0.40	0.82	15.1	+	+
S61	++	++	13.1	3.8	0.81	0.68	0.97	17.2	+	+
S22	++	++	15.7	4.9	0.97	0.52	0.81	15.3	+	+
S. typhimurium 1098	++	++	17.2	5.1	0.97	0.60	0.82	19.1	+	+
A. cloacae 5920	++	++	21.1	6.1	0.99	0.49	1.21	22.0	+	+
S56	±	++	10.1	3.1	0.73	0.41	0.072	12.8	-	-
S22M	-	++	9.8	2.8	0.81	0.38	0.051	10.2	-	-
CA10R	-	++	12.2	4.1	0.74	0.42	0.047	9.7	-	-
CA10	-	-	9.8	5.1	-	0.41	0.052	8.7	-	-
CA 3	-	-	10.1	3.9	0.81	-	0.091	12.2	-	-
S23	-	-	14.1	3.1	0.91	0.62	0.14	13.1	+	+
S. typhimurium 1542	-	-	9.8	3.8	0.61	0.21	0.072	15.3	-	-
S. typhimurium 1543	±	±	10.2	2.9	0.58	0.17	0.096	12.8	-	-

Organism	Mucoid on ordinary medium	Mucoid on PFA	PGI	PGM	UDPG PP	ENIM	DEHYD	PMI	GDPM PP	GDPF SYN
S53/1	-	-	10.1	3.1	0.58	0.39	0.62	7.9	+	+
S53/2	-	-	9.7	2.8	0.72	0.28	0.47	10.1	+	+
S53/3	-	-	12.8	2.1	0.69	0.33	0.51	8.9	+	+

- not detectable.

Activity expressed as μM substrate reappearing or product appearing/mg. protein/min.

UDPG-4-Epimerase, phosphomannose isomerase, and GDPF synthetase.

Clearly the levels of certain enzymes in mucoid strains of K12 and other Colanic acid synthesising strains are of interest when compared with those of strains which do not normally produce Colanic acid under normal conditions, if at all.

Enzyme assays were performed on cell-free extracts of several strains grown in broth and the results are outlined in Table 15. As indicated previously, the enzymes GDPM pyrophosphorylase and GDPF synthetase were indicated simply as being present or absent within the limits of the detection methods. All preparations were from stationary-phase cells.

It can be seen that there is very little difference between mucoid cells and non-mucoid cells in the levels of the enzymes phosphoglucose isomerase, phosphoglucomutase, UDPG pyrophosphorylase and UDPG-4-Epimerase. The most striking feature to emerge from the results is the difference in the levels of the enzymes in the GDPF pathway and the level of UDPG dehydrogenase in mucoid strains with a few notable exceptions. It seems quite clear that highly mucoid strains produce some 5-10 times more UDPG dehydrogenase than non-mucoid strains, and that the level of phosphomannose isomerase in mucoid strains is 50-100% increased over the level in non-mucoid strains. Furthermore, the enzymes GDPM pyrophosphorylase and GDPF synthetase are only detectable in mucoid strains with the methods available.

Strains CA3 and CA10 are clearly similar to normal K12 derivatives such as S56 and S22M which do not normally produce Colanic acid, apart from their single enzyme defects.

The S53 derivatives S53/1, S53/2 and S53/3 have enzyme levels not markedly different from S53 and other mucoid types although UDPG dehydrogenase and phosphomannose isomerase are down in activity about 30%. However, GDPM pyrophosphorylase and GDPF synthetase were detectable, clearly these three strains are not non-mucoid by reason of a defect in sugar nucleotide metabolism.

Salmonella strains 1542 and 1543 resemble CA10, S56 and others of this type in that UDPG dehydrogenase is at low level, and GDPM pyrophosphorylase and GDPF synthetase are not detectable, despite the fact that 1543 does produce small amounts of Colanic acid.

S23 is clearly different from the rest in that although UDPG dehydrogenase is at low level, the GDPF pathway enzymes would appear to be at the same level as those of mucoid organisms.

EXAMINATION OF LIPID EXTRACTS

In view of the evidence for lipid-linked oligosaccharide intermediates which participate in the biosynthesis of other bacterial heteropolysaccharides, it was decided to examine the lipids of a typical highly mucoid strain, S53, to determine if

such compounds could be detected.

Stationary-phase cells were grown in broth in shake culture overnight at 30°, the cells were spun down at 0° and 20,000 g. The cell paste was treated in either of three ways:

(a) Extracted directly with 3 volumes chloroform/methanol 3/1 (v/v) for 15 min. with stirring. The debris was centrifuged at 10,000 g, and the extraction repeated. The pooled supernates were concentrated to dryness on a rotary evaporator at 20°, and the residue dissolved in a small volume n-butanol, and kept at -20°.

(b) Made up in a small volume ice-cold saline, and subjected to ultrasonic disintegration for 5 min. The broken cell preparations were then extracted twice with 3 volumes n-butanol with stirring for 15 min., centrifuging the debris at 10,000 g each time. The pooled butanol layers were concentrated to small volume and kept at -20°, clarifying if necessary by centrifugation at 20,000 g.

(c) Made up in a small volume of ice-cold saline, and extracted with 3 volumes boiling ethanol for 10 min. with stirring. The debris was centrifuged at 5,000 g, and the supernate taken to dryness on a rotary evaporator at 20°. The residue was extracted twice with a small volume of n-butanol by shaking vigorously for 15 min., the butanol layers clarified by centrifugation at 20,000 g, pooled, and kept at -20°.

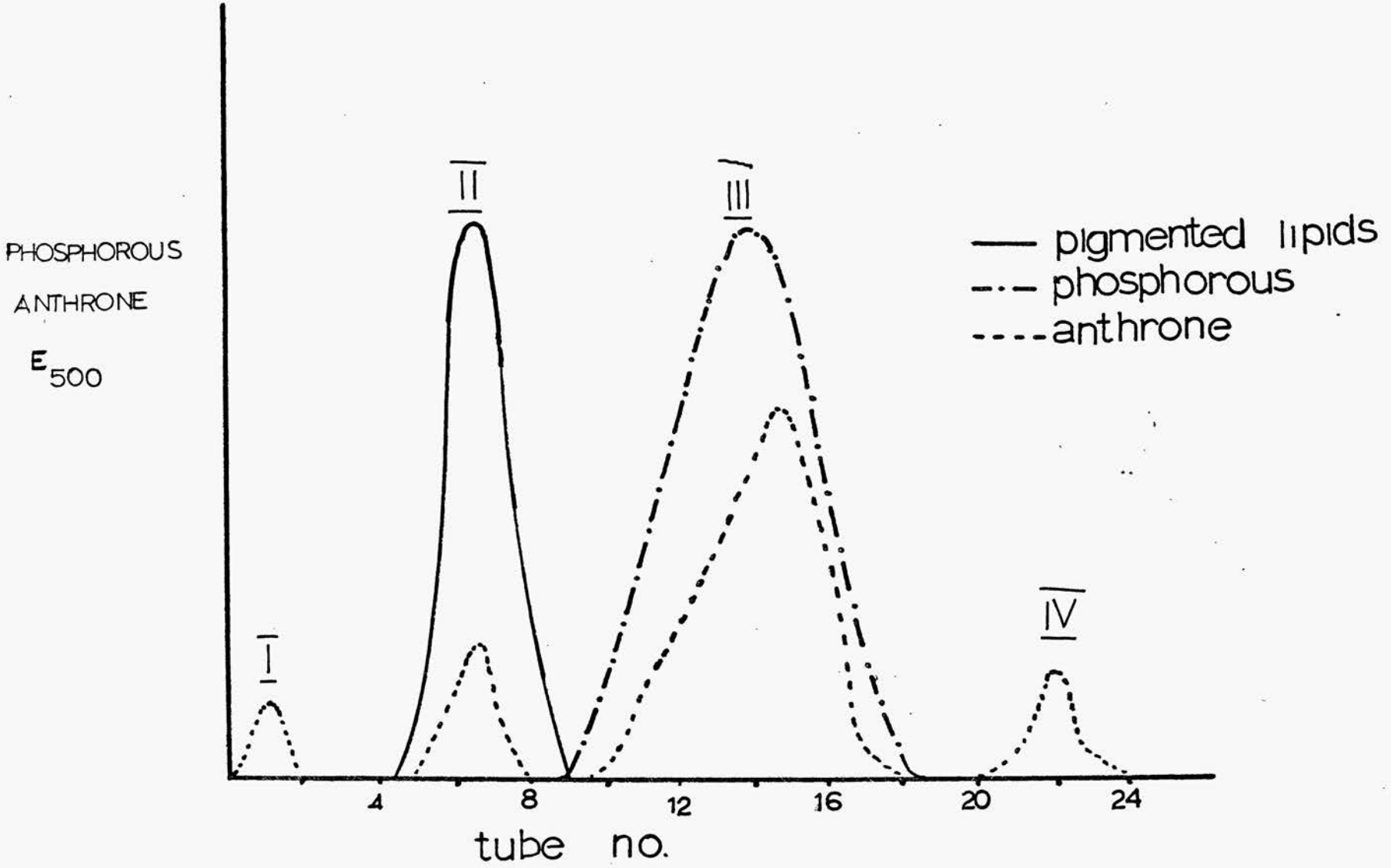
Procedures (a) and (b) were also carried out with cell pastes which were suspended in a small volume 0.02 M phosphate buffer pH 7.2 containing 2% (w/v) glucose, at 30° for 5 min., in order to try and enhance polysaccharide synthesis.

One of the problems involved in lipid extractions is that considerable quantities of hydrophilic compounds, notably free sugars, contaminate the preparations, and attempts to remove them by washing with water, as advocated by many workers, results in considerable quantities of lipids, mainly phospholipids, passing into the aqueous phase. Accordingly other methods were used to remove hydrophilic material.

A 25 x 1 cm. column of Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden) was prepared from distilled water under gravity, and equilibrated with distilled water at 7 ml./hr. n-Butanol was then pumped through at the same rate, until the void volume of the column had passed through, determined by the volume of water displaced. Lipid extract was then applied to the column, washed in with n-butanol, and pumping continued at the same rate until a further void value was eluted. This fraction was taken to dryness on a rotary evaporator at 20°. Under these conditions, contaminating hydrophilic material is retained in the column within the Sephadex particles, whereas the lipid material does not enter the particles, and is eluted in the void volume.

Chromatography of such fractions was carried out on thin-layer silica of 0.3 mm. thickness prepared from Whatman SG 41

FIGURE 34 CHOMATOGRAPHY OF S53 LIPIDS ON LH-20 SEPHADEX



Silica Gel, in Solvent F, to determine any qualitative differences in the lipids extracted by the three different methods, but no differences were detected, on visualisation of the lipids with iodine vapour.

Eluates from G-25 columns were dissolved in a small volume chloroform/methanol 3/1 (v/v) and applied to a 25 x 1 cm. volumn of Sephadex LH-20, prepared according to the method of Maxwell and Williams (1967). Chloroform/methanol 3/1 (v/v) was pumped through at 7 ml./hr. and 1 ml. fractions were collected. The fractions were examined for phosphorus and total sugar, and fractions were pooled accordingly. Figure 34 illustrates the result of one such experiment.

It was found that the small amount of carbohydrate material eluted in Peak I was probably material eluted from the column itself, and was not lipid judging from its behaviour in Solvent F and its lack of staining with iodine vapour. Similarly Peak IV is contaminating hydrophilic material which still remains even after treatment on the Sephadex G-25 column. Peaks II and III, which probably represent the neutral lipids and phospholipids of the cell respectively, were concentrated and examined for oligosaccharide phosphate derivatives of the type found in LPS and murein biosynthesis. This was accomplished by treating the fractions with 45% (w/v) phenol at 65° after the method of Weiner et al. (1965) and hydrolysis in 0.07 N NaOH as described by Dankert et al. (1966), in order to try and remove any oligosaccharide phosphates from the lipid

material into the aqueous phase. The aqueous layers in both cases were then subjected to electrophoresis in Buffer A at 80-100 mA for 6 hr., and the electrophorerogram stained for phosphorus and sugars.

Despite many attempts involving lipid material from up to 40 g. dry weight of cells, no such compounds were detected. In case the compounds were so labile that the extraction procedures were causing the release of oligosaccharide material into the aqueous layer, the lipids were extracted with boiling ethanol using method (c) and the ethanol layer reduced to dryness, then redissolved in a small volume distilled water. This material was hydrolysed with 0.01 N HCl at 100° for 20 min. to split any sugars from nucleotide derivatives, and then subjected to electrophoresis at 80-100 mA for 6 hr. After staining for phosphorus, and elution of phosphorus containing areas, followed by hydrolysis in 1.0 N H_2SO_4 for 1 hr., the only sugars which were detected in areas removed from the origin, were glucose, mannose and glucuronic acid. The glucose and mannose presumably are there as sugar phosphates, normal components of the soluble pool, and glucuronic acid has probably been liberated from UDPGA and moved from the origin because it is an acid.

Since S53/1/2/3 contained all the necessary nucleotide sugars but were still non-mucoid, it was reasoned that these cells must have a block in exopolysaccharide biosynthesis after the nucleotide sugar stage, and if lipid-linked intermediates

existed, then the block may result in accumulation of such compounds. However, extraction of lipids from these strains and treatment as previously described, did not result in the detection of such compounds.

PREPARATION OF ^{14}C-LABELLED LIPIDS

In order to try and label small amounts of lipid-linked intermediates which might be present, S53 cells were grown in shake culture overnight at 30° in YE medium to give maximum polysaccharide production. The cells were centrifuged at 50,000 g, and washed with saline to remove as much polysaccharide as possible. The cells from 1 l. were uniformly resuspended in 200 ml. of YE medium devoid of nitrogenous material, and reincubated with gentle shaking at 30° for 1 hr. After this time 20 ml. 10% (w/v) glucose was added, followed by 20 μC uniformly labelled ^{14}C-glucose. After periods ranging from 30 min. to 3 hr. and at incubation temperatures of 30° and 15°, lipids were extracted and purified as previously described.

Generally it was found that under these conditions less than 1% of the total radioactivity added was incorporated into lipids as determined by the material eluted from a Sephadex G-25 column. The bulk of the radioactivity incorporated went into the phospholipids, as determined by chromatography on Sephadex LH-20, but after phenol extraction and electrophoresis of the aqueous layer, no labelled material with the electro-

phoretic mobility of oligosaccharide or sugar phosphates was detected from any of the experiments.

ATTEMPTS TO SYNTHESISE COLANIC ACID IN CELL-FREE SYSTEM

Various methods were used to prepare an enzyme system from S53, notably the methods employed by various workers to achieve biosynthesis of LPS in cell-free system. Included were the EDTA lysate method of Dankert *et al.* (1966), and the cell-envelope preparation of Weiner *et al.* (1965). Other preparations were simple broken cell preparations achieved by ultrasonic disintegration, and various particle size preparations from such disrupted cells, including the 10,000 *g* and the 20,000 *g* sediments.

A typical reaction mixture after Dankert *et al.* (1966) consisted of: 100 µl. 1.0 M Tris-HCl pH 7.8; 10 µl. $MgCl_2$; 10 µl. 0.1 M UDPG; 10 µl. 0.1 M GDPM; 10 µl. 0.1 M UDPGal; 10 µl. 0.1 M UDPGA; 300 µl. H_2O; 500 µl. enzyme preparation; 1 µl. (0.1 µC) ^{14}C-UDPGal, ^{14}C-UDPGA or ^{14}C-UDPG.

Since GDPF was not available, GDPM was included in its place in the hope that the enzymes responsible for the biosynthesis of Colanic acid might have their specificity altered sufficiently by the method of preparation to allow some incorporation of mannose in place of fucose, in the particulate preparations. In the broken cell preparations, GDPF synthetase is active, and 20 µl. 0.02 M NADPH was added to stimulate this

enzyme, and bring about appreciable synthesis of GDPF.

After incubation the reaction was stopped by the addition of an equal volume 90% (w/v) phenol at 65°. Extraction with phenol was carried out for 10 min. at 65° and the aqueous layer subjected to electrophoresis in Buffer A at 80-100 mA for 4 hr. Under these conditions any oligosaccharide units synthesised or any high molecular weight material synthesised will run slower than the nucleotide sugars in the reaction mixture or not move from the origin depending on size and charge. The electrophorerogram was cut into strips and counted.

Despite many attempts with the various enzyme systems at various temperatures from 15-37° and at pH ranging from 6.5 - 8.5, with ^{14}C-UDPG, ^{14}C-UDPGal, and ^{14}C-UDPGA, in the presence of the four nucleotide sugars believed to be precursors, no incorporation of label was observed into any material whether small molecular weight or large molecular weight.

Extraction of the incubation mixtures at various times with chloroform/methanol 3/1 (v/v), or n-butanol, also failed to detect any radioactivity passing into the lipid phase.

DISCUSSION

The Family Enterobacteriaceae consists of no fewer than five Tribes and ten Genera (Bergey's Manual of Determinative Bacteriology, 7th Edition, 1957), including the well investigated Escherichia, Salmonella, Shigella, and Klebsiella groups, but also encompassing the less well known Erwinia, Serratia, and Aerobacter groups. It has been said (Lüderitz, Jann and Wheat, 1968) that Colanic acid is likely to be the M or mucus antigen of the Enterobacteriaceae, but clearly, since the original serological detection of the antigen was only in the Salmonella-Escherichia group, the statement is presumably not meant to be extrapolated to suggest that Colanic acid can be found as a characteristic antigen in all groups of the Enterobacteriaceae. It is likely that the authors have considered only the Shigella-Salmonella-Escherichia group of intestinal commensals and pathogens, as the true enterobacteria, and not the entire Enterobacteriaceae per se. If so then the statement bears more relationship to the situation.

Previous studies have indicated that Colanic acid, or material of that chemotype has been isolated from several Escherichia strains, including the K12 line (Goebel, 1963; Sapelli and Goebel, 1964; Markowitz, 1964), several Salmonella strains (Anderson and Rogers, 1963), and one strain in the Paracolobactrum group (Anderson and Rogers, 1963), which is intermediate between Salmonella and Escherichia, and at various

times has been classified with one or the other. There is also one report of material of this type being isolated from the Aerobacter group (Sutherland and Wilkinson, 1965). Markowitz (Kang and Markowitz, 1966) has further indicated that many K12 strains normally considered to be non-mucoid, can be induced to become mucoid when grown in the presence of p-fluorophenylalanine (PFA). The exopolysaccharide produced under these conditions is identical to that produced by normally mucoid strains of K12, of the same chemotype as Colanic acid. The inference is that all strains of K12 possess the genetic information for the synthesis of Colanic acid, but that the vast majority have this ability repressed by a regulator gene, normally mucoid strains being considered as derepressed mutants.

The results obtained in this investigation, on the production of Colanic acid by various K12 strains and other members of the Shigella-Salmonella-Escherichia group, verify the results of Kang and Markowitz (1966). It seems certain that, with the exception of those strains which have a defect in a gene coding for a portion of the synthesis of the molecule, all K12 strains have the genetic ability to synthesise Colanic acid. Most strains are normally non-mucoid, but such strains can be induced to form exopolysaccharide by growing them in the presence of PFA. The exopolysaccharides produced by the various strains have been identified by chromatographic analysis, but also by the use of specific phage-induced depolymerase enzymes which have been shown to be active only on

Colanic acid.

These results have been extended to indicate that much the same situation exists within the genus Salmonella. Many of these strains are normally mucoid (Anderson and Rogers, 1963) producing material of the same chemotype as Colanic acid, and for the first time this material has been shown to be identical with that produced by K12 strains, by typing the expolysaccharide with the specific depolymerases. A considerable number of other Salmonella strains, previously considered non-mucoid, have been shown to produce an exopolysaccharide, identified as Colanic acid by the same criteria, when grown in the presence of PFA.

Several other strains which did not appear to produce exopolysaccharide on solid media, even in the presence of PFA, have been shown to produce exopolysaccharide material after long incubation in liquid medium containing PFA. The material isolated in all cases gave a positive colour reaction for uronic acid, strongly suggesting that this material was exopolysaccharide in origin since uronic acids are seldom, if ever, found in any other type of polysaccharide in the bacterial cell (Lüderitz, Jann and Wheat, 1968). On hydrolysis in all cases the material was shown to contain the monosaccharides found in Colanic acid. Other sugars were present, a reflection of cell lysis, and contamination by other cell polymers over the long incubation period. This in itself is hardly conclusive evidence for the synthesis of Colanic acid, but, taken in conjunction with the evidence that a considerable number of strains

synthesise Colanic acid, identified by other criteria, on ordinary media or in the presence of PFA, this is strong circumstantial evidence that the material synthesised is Colanic acid. All the Salmonella strains with the exception of S. typhimurium SL 1542, which is a special case and will be considered as such, appeared to synthesise material on normal media or in the presence of PFA which was identified as Colanic acid by its sensitivity to depolymerase enzymes, or material which on hydrolysis gave the characteristic monosaccharide components of Colanic acid. S. typhi is also a special case in that this strain is known to produce an extracellular polymer of N-acetyl-galactosaminuronic acid, so in this case a colour reaction for a uronic acid does not necessarily indicate the presence of Colanic acid, and it is likely that the monomer sugar would have a similar chromatographic mobility to glucuronic acid, in the solvents used. However, in this case the presence of fucose is probably significant enough to indicate that Colanic acid is present. These findings seem to indicate that a similar pattern of genetic capability with respect to the synthesis of Colanic acid, exists within the Salmonellae as within the K12 strains, the normal example of a Salmonella strain being non-mucoid and therefore having exopolysaccharide synthesis repressed, but capable of being derepressed in the presence of PFA.

Colanic acid chemotypes have been described several times within the Escherichia group, outwith the K12 strains, sometimes

in conjunction with separate capsular antigens (Ørskov et al., 1963). Recently (I.W. Sutherland, unpublished results), 18 out of 21 different strains of Escherichia have been shown to produce Colanic acid on ordinary media, some only after incubation at low temperature. Whether or not the remaining 3 strains could be induced to form Colanic acid in the presence of PFA remains to be investigated, but this seems strong evidence to suggest that all members of the genus Escherichia have the genetic ability to form Colanic acid, and a similar pattern of repression and depression exists as with K12 strains and the Salmonellae.

The genus Shigella is probably closest to the Salmonella-Escherichia group, but there are no reports of mucoid strains being isolated. Attempts to get strains of Shigella flexneri to synthesise Colanic acid on various types of media, including growing the cells in the presence of PFA, all met with failure. Of the other genera within the Enterobacteriaceae, the most extensively examined is the capsulate Klebsiella group. Despite the large amount of information available about the capsular polysaccharides of the group (Table 4), and the extremely varied compositions reported, only on one or two occasions has material with the same chemotype as Colanic acid been detected. One of these strains which produces a capsular polysaccharide of the same chemotype, Klebsiella type 1, has been investigated. It has been found that although the exopolysaccharide contains glucose, galactose, fucose, and

glucuronic acid, it is not sensitive to the specific depolymerases which have been used to identify Colanic acid. Clearly this polysaccharide is not Colanic acid, and in view of the infrequency of the reports of Colanic acid chemotypes within the group, it seems likely that Colanic acid is not produced by members of the group. Other genera such as Erwinia and Serratia have not been investigated extensively. Exopolysaccharides are produced, particularly by Serratia (Table 4), but the information available does not include the report of any Colanic acid chemotypes.

In general it seems that Colanic acid is restricted to the Salmonella-Escherichia group, and a few intermediate types such as Paracolobactrum. The report of Colanic acid being synthesised by Aerobacter cloacae (Sutherland and Wilkinson, 1965) at first sight appears unusual in that the Aerobacter group has been previously included in the Klebsiellae, some strains having variously been called Aerobacter aerogenes, Klebsiella aerogenes, and Klebsiella pneumoniae. However, it now seems certain that Aerobacter cloacae is quite different from the Klebsiella group, both in biochemical characteristics and habitat, and at various times has been placed in the separate genus Cloaca.

Significantly, all of the strains reported to synthesise Colanic acid, including Aerobacter cloacae, are normal inhabitants of human and animal intestines. The other members of the Enterobacteriaceae, outwith the Salmonella-Escherichia-

Shigella group, such as members of the Klebsiella, while some may be human pathogens and commensals, are not commonly encountered in the intestine. In view of the rapid and easy exchange of genetic material between Escherichia and Salmonella, it may be that the genetic information for the synthesis of Colanic acid is readily transferable amongst members of the Enterobacteriaceae normally resident in the intestines of man and animals. Recently Hardy and Nell (1967) have demonstrated the rapid transfer of several factors including mucoidness, amongst members of the Escherichia. The rapidity of transfer suggested some extrachromosomal factor, and the authors were able to transfer some of the factors to strains of Sh. flexneri, but the mucoid character could not be transferred to this strain. The nature of the mucoid material was not investigated, but it seems likely to be Colanic acid. Precisely what is being transferred in this system is also not clear as yet, and it may be some type of regulator gene as described by Markowitz (Markowitz and Rosenbaum, 1965), or possibly a small region of genetic material containing some of the key enzymes involved in Colanic acid synthesis.

Precisely what makes an exopolysaccharide slime or capsulate also remains to be investigated. One of the strains investigated, S53C, produced capsules of Colanic acid. The change from slime to capsule must be very subtle, perhaps involving the tertiary structure of the polymer, since the depolymerase enzymes, despite their high specificity, act on both

slime and capsule types of polymer. The strain S61 is also of interest, since the same conclusions apply in this case, the exopolysaccharide produced being sensitive to the depolymerase enzymes, but quite clearly different in its physical properties, being extremely viscous and rubbery. The possibility that there might be some subsidiary groupings in the exopolysaccharide which alter its physical properties has been examined. The most obvious example of such a group is an acyl group of some type, probably acetyl, since acetyl groups are known to be extremely important antigenically, and they are often lost by purification procedures. Analyses have been performed on the exopolysaccharides of S53, S53C, and S61 (I.W. Sutherland, unpublished results), and O-acetyl groups have been detected by the method of Thompson (1951) in all three. However, no appreciable difference in the quantity of acetyl present in each was detected, and the problem of slime and capsule remains to be more fully investigated.

As to how the phage-induced depolymerase enzymes act on the exopolysaccharides has also been considered. In one instance (Sutherland, 1967) a phage enzyme acting on Klebsiella type 54 has been found to hydrolyse the polymer into small molecular weight oligosaccharides which have been characterised. However, phage-induced depolymerases for Colanic acid have been previously reported to lower the viscosity of the polymer without releasing any small molecular weight fragments (Sutherland and Wilkinson, 1965). The enzymes prepared from the

phages provided, fall into this category, and examination of the enzymes prepared from all of the phages isolated, indicated that they too were of this type. The possibility of deacetylation by the depolymerases, causing changes in the secondary structure, was considered, but examination of exopolysaccharides before treatment with depolymerase, followed by examination after treatment with depolymerase and dialysis, revealed the same acetyl composition in both (I.W. Sutherland, unpublished results). Presumably the effect of the phage enzymes is on some linkage between chains of polysaccharide, or on some other grouping not yet investigated.

Examination of the nucleotide pools of certain normally mucoid and non-mucoid strains of K12 and Salmonella has revealed certain differences between the nucleotide sugars to be found in both. With the exception of strains S23, CA3, CA10, S53/1, S53/2, S53/3 and S. typhimurium SL 1542 and 1543, which are special cases and will be considered as such, the difference in nucleotide sugar pattern between mucoid and non-mucoid cells was quite significant. In particular mucoid cells contained large amounts of UDPGA, sometimes up to fifty times the level to be found in non-mucoid cells, and the level of UDPG dehydrogenase in mucoid cells was some 10 to 20 times higher than in non-mucoid cells.

Also present in the soluble pool of mucoid cells is GDPF, and the enzymes concerned in its synthesis, GDPM pyrophosphorylase, and GDPF synthetase, can be detected with the methods

available, whereas in non-mucoids all are undetectable. It is possible that non-mucoids may have low levels of these, in the same way that non-mucoids have low levels of UDPGA and UDPG dehydrogenase, since the assay methods used are not very sensitive, but at least it can be said that mucoid cells have much higher levels of GDPF, GDPM pyrophosphorylase and GDPF synthetase.

The comparison of these sugar nucleotides and enzymes in non-mucoid and mucoid cells very strongly suggests their incrimination in Colanic acid synthesis.

Both mucoid and non-mucoid cells had UDPG and UDPGal in the soluble pool at substantially the same level. Since all the strains investigated, with the exception of Aerobacter cloacae NCTC 5920, have glucose and galactose in the LPS, the presence of these sugar nucleotides is to be expected since they have been shown many times to be the precursors in glucose and galactose transfer to LPS. The absence of any other nucleotide derivatives of these sugars in the soluble pool does not necessarily mean that both LPS and Colanic acid have the same glucose and galactose nucleotide precursors, but since the mucoid A. cloacae has no galactose in the LPS, but has UDPGal in the soluble pool, this is strong circumstantial evidence for their involvement in Colanic acid synthesis.

These differences in nucleotide sugar pattern and certain enzyme levels between mucoid and non-mucoid strains strongly support the pathway outlined in Figure 33 as being operative in Colanic acid biosynthesis.

dTDPRh was also detected in all the strains investigated, presumably a reflection of its function as a precursor in LPS synthesis. In particular, the level of dTDPRh found in S53 is relatively high, and it is likely that other K12 strains have similar levels. K12 is said to be a semi-rough strain of E. coli (Rapin and Mayer, 1966) with no detectable O-antigens, and it is possible that the original smooth strain from which it was derived contained a higher proportion of rhamnose in the LPS, and perhaps other sugars in addition to those found in K12. It has been shown previously that in some strains of E. coli, dTDPRh accumulates to a level similar to that found in S53 (Nikaido and Nikaido, 1964) but conversely other strains have been shown to possess the necessary enzymes for its synthesis, without any dTDPRh being detectable in the soluble pool, and (Okazaki, Strominger and Okazaki, 1963) the difference between the two is attributed to a feedback inhibition mechanism being operative in one case.

Since O-acetyl groups are known to be present in Colanic acid, the question arises as to when these substituent groupings are inserted. As far as is known, no O-acetylated sugar nucleotides were isolated during this investigation, but since O-acetyl groups are extremely labile, it may well be that such groups would be lost during isolation and identification procedures. In particular, the procedure involving hydrolysis in 0.01 N HCl, prior to identification of the nucleoside diphosphate and sugar released, may well liberate O-acetyl groups

present on the sugar moiety. No reports have been made of the isolation of O-acetylated sugar nucleotides perhaps because of these reasons and, in contrast to N-acetylation, there is little information available as to how and when the acetyl groups are inserted. The evidence there is suggests that the insertion occurs at the polysaccharide level, and the Robbins group (Robbins et al., 1966; Keller, 1966) have demonstrated transfer of acetyl groups from Acetyl CoA to the LPS of S. anatum in a cell-free system. In contrast Sutherland and Wilkinson (1968) have shown that acetylation of this type in the exopolysaccharide of a Klebsiella aerogenes strain appeared to occur at highest efficiency in a cell-free system with a glucuronosyl-galactose disaccharide isolated from the exopolysaccharide, which led these authors to suggest that O-acetylation might occur at the level of a lipid-linked oligosaccharide intermediate. However, further work is required to establish unequivocally the stage at which O-acetyl groups are attached.

Given the genetic potential for the synthesis of Colanic acid, then the question arises as to how the synthesis of the polymer is controlled, since it has been demonstrated that many strains of the Salmonella-Escherichia group have the ability to synthesise Colanic acid, but do not do so under normal conditions.

In general terms the control of polysaccharide synthesis can be either metabolic or genetic. The first possibility can be brought about in several ways, either by affecting the rate of the final transferase reaction in polysaccharide biosynthesis,

or by affecting the rate of formation of precursors involved in the pathway. The final transferase reaction is involved in the control of glycogen synthesis in yeast and mammals, but control of this type has been documented in only a few systems, and this may be a reflection of the relative simplicity of the systems where it is known to happen, and the nature of the sugar nucleotide precursors involved. In yeast and mammalian glycogen synthesis the nucleotide sugar donor is UDPG, and, since UDPG is involved in many other reactions in the cell, it would be undesirable if the lowering of glycogen synthesis brought about a concomitant lowering of the UDPG level in the cell, so in this case the control system has to be at the final stage. The other factor is that the polymer synthesised is a homopolymer, and thus only one enzyme requires to be controlled. In the case of a heteropolymer the situation is more complex, with several transferase enzymes participating, and further complexities arise if an intermediate lipid-linked stage is involved, so it is likely that control would occur at an earlier stage. Where the sugar nucleotide precursor or precursors are unique to a particular pathway, then the control can be applied at this level. As Ginsburg (1964) has discussed, the existence of different nucleotide derivatives of the same sugar is an important factor, since it allows separation of biosynthetic pathways, and therefore easier control. The method of control can be by feedback inhibition, stimulation of enzymes in much the same way as the stimulation of mammalian

glycogen synthetase, or by specific removal of precursors through the action of degradative enzymes. Feedback inhibition of enzymes involved in the biosynthesis of sugar nucleotides is well documented in the bacteria, particularly in the systems involved in the biosynthesis of deoxy and dideoxy sugars. In general terms the end product inhibits the first enzyme reaction unique to its biosynthetic pathway. Such mechanisms presumably explain why nucleotide sugar intermediates such as nucleoside diphosphate 4 keto-6 deoxysugars are seldom encountered in nucleotide pools unless a pathological situation exists (Ginsburg, 1964). Many examples exist of such feedback inhibition systems, e.g. the inhibition of dTDPG pyrophosphorylase by dTDPG and dTDPRh (Berstein and Robbins, 1965; Melo and Glaser, 1965); the inhibition of CDPG pyrophosphorylase by CDP paratose (Mayer and Ginsburg, 1965). The fineness of control of such a system has been demonstrated by Kornfeld and Glaser (1966) in their study of the synthesis of GDPM and GDPF, where they have illustrated how the rate of synthesis of two nucleotide sugars, with the same precursors, can be controlled independently by feedback inhibition. It has been considered that feedback inhibition is one of the reasons why many strains do not accumulate uridine nucleotides in the presence of penicillin, and why many R strains of Salmonella do not accumulate nucleotide derivatives of type specific sugars (Mayer and Ginsburg, 1964). There is also some evidence that stimulation of nucleotide sugar pyrophosphorylases may occur by compounds

of intermediary metabolism. Shen and Preiss (1965) have shown that certain glycolytic intermediates will stimulate bacterial glycogen synthesis, and the effect has been traced to a stimulation of ADPG pyrophosphorylase. This system has similarities with the mammalian system, and again this type of control has not been widely reported, possibly for the same reasons that apply to glycogen synthetase systems. Specific removal of nucleotide sugars may occur through the mediation of degradative enzymes, but this aspect is less well established as a control mechanism. Glaser (1965) has shown a CDP glycerol pyrophosphatase in extracts of a Lactobacillus, and has suggested that this might be a control mechanism in teichoic acid synthesis. Other enzymes which degrade different sugar nucleotides are known (Cabib and Carminetti, 1961; Dankert, Concalves and Recondo, 1964), but these have been discussed by Glaser, Melo and Paul (1967) along with several new types which they detected, and in view of their location in a position on the outside of the cell it has been suggested that they may simply be enzymes which make nucleotide sugar compounds found in the environment, available to the cell.

The best documented pieces of evidence for the genetic control of enzymes involved in polysaccharide biosynthesis are the elegant studies of the Robbins group on the lysogenic conversion of certain strains of Salmonella. Introduction of a phage genome has been shown to depress the enzymes concerned in the O acetylation of LPS, and the addition of galactose in an α

linkage to the LPS (Robbins and Uchida, 1965a; 1965b). The α galactosyl residues were further shown to be replaced by β galactose residues under the direction of an enzyme coded for by the phage genome (Robbins et al., 1965). Similar results were shown in a different system where the specific transferase concerned in the formation of a galactosyl α 1-4 mannose linkage in LPS was depressed, and replaced by another transferase which catalysed the formation of a galactosyl α 1-6 mannose linkage, on introduction of a phage genome (Bagdian, Luderitz and Staub, 1966).

In the situation under investigation, the non-mucoid strains, again with the exception of S23, CA3, CA10, S53/1, S53/2, S53/3 and the two S. typhimurium strains SL1542 and SL1543, have very low levels of UDPGA, and undetectable GDPF. This strongly suggests that the control of Colanic acid synthesis lies at the nucleotide sugar level, since if it were at the transferase level, then the levels of those sugar nucleotides found believed to be involved in Colanic acid biosynthesis would not be expected to be lower than those found in mucoid cells. The fact that the enzymes involved in the synthesis of these sugar nucleotides are at a very much lower level in non-mucoid cells, rules out the possibility of feed-back inhibition taking place in some way or the degradation of sugar nucleotides, and in view of the complexity of the system, it is unlikely that the control mechanism is similar to that involved in bacterial glycogen synthesis. This suggests that control

of synthesis of GDPF and UDPGA is at the genetic level, complementing the suggestion of Markowitz (1964) that the synthesis of GDPF, and to a lesser extent UDPGal, was under genetic control in non-mucoid strains. In this investigation the levels of UDPGal and UDPG-4-Epimerase were not found to be sufficiently different in mucoid and non-mucoid strains to suggest a similar situation. Markowitz (Kang and Markowitz, 1966) had further suggested that the genetic control in non-mucoid strains could be altered by growing cells in the presence of PFA, under which conditions the cells became mucoid, this being taken to mean that a repressor molecule controlling Colanic acid synthesis becomes altered, allowing several enzymes to become derepressed. Several strains of K12, non-mucoid under normal conditions, could be induced to become mucoid by growing them in the presence of PFA, and the nucleotide pools of such strains grown under these conditions have been investigated. In all cases the level of UDPGA to be found in the presence of PFA was very much higher than when the cells were grown in broth, and approached that of normally mucoid strains. In all cases examined, GDPF became detectable in the soluble pool, and in general terms the nucleotide pool of such strains grown in the presence of PFA resembled that of normally mucoid strains, again complementing the results of Markowitz (Kang and Markowitz, 1966), which indicated that the enzyme GDPF synthetase increased in activity when such cells were grown in the presence of PFA. The non-mucoid strain S22M

is of interest since it was derived from S22, a highly mucoid strain which has high levels of UDPGA, GDPF, and the enzymes required for their synthesis. S22M has low levels of UDPGA, and no detectable GDPF, but, in the presence of PFA, produces levels of these nucleotide sugars similar to S22, and the enzymes involved increase accordingly. In this instance it seems that a derepressed strain has mutated to become repressed.

Several strains investigated are different from the mucoid and non-mucoid strains discussed, by virtue of an enzyme defect which in some cases is known, but in others can only be inferred.

K12 Strains CA3 and CA10 are known mutants, and do not produce Colanic acid under any circumstances. CA3 is a UDPG-4-Epimerase-less strain, and, as might be expected, this enzyme is not detectable, and no UDPGal is found in the nucleotide pool. However, low levels of UDPGA are detectable, similar to the non-mucoid strains already discussed, and no GDPF is detectable. When grown in the presence of PFA, the level of UDPGA is markedly increased and GDPF becomes detectable in the nucleotide pool, suggesting that this strain is a normal K12 non-mucoid strain with a single enzyme defect. K12 strain CA10 is a UDPG pyrophosphorylase-less mutant, and might be expected to lack UDPG, UDPGal, and UDPGA in the nucleotide pool. However, low levels of these nucleotide sugars were detected, suggesting that the mutation is leaky, or that revertants are thrown off at a high rate. The latter seems to be the case, since galactose-positive colonies can be easily obtained, and one of

such has been purified and designated CA1OR. When CA1O is grown in the presence of PFA, GDPF becomes detectable in the nucleotide pool, suggesting that this again is a normal non-mucoid strain of K12 with a single enzyme deficiency. This was confirmed since the revertant CA1OR is a typical non-mucoid strain of K12, which can be induced to form Colanic acid in the presence of PFA. These results indicate that CA3 and CA1O have the necessary genetic information to synthesise Colanic acid, apart from single enzyme deficiencies, but that they are typical depressed non-mucoid strains. This confirms the postulated involvement of UDPG and UDPGal in Colanic acid biosynthesis. An interesting aspect of the investigation of the nucleotide pools of CA3 and CA1O, is that while dTDPRh can be detected in CA3, CA1O has very low levels, which is strange since both strains are essentially similar apart from their enzyme defects. The lack of dTDPRh in CA1O could be due to dTDPG being used in place of UDPG for some reactions, or it could be that the mutation in UDPG pyrophosphorylase also affects dTDPG pyrophosphorylase. The revertant CA1OR has dTDPRh in the soluble pool at much the same level as CA3 as far as was ascertained, and it may be that this particular mutation does affect dTDPG synthesis as has been described by Wu (1966).

S53/1, S53/2, and S53/3 are probably all identical and are unusual in that they do not produce Colanic acid under any circumstances. Examination of the nucleotide pools indicates that in all three cases the nucleotide pool is substantially

similar to S53, with high levels of UDPGA and detectable GDPF. Clearly these strains must have a block in Colanic acid synthesis somewhere after the nucleotide sugar level. The nucleotide sugars involved in Colanic acid synthesis do not accumulate beyond the levels normally found in normally mucoid strains, and the levels of UDPGA are slightly reduced, indicating that some feed-back control mechanism is operating.

K12 strain S23 does not produce Colanic acid under any circumstances, and examination of the nucleotide pool indicates that while GDPF, UDPG, and UDPGal are present at substantially the same level as in mucoid strains, UDPGA is present at very low level and the enzyme UDPG dehydrogenase is also at low level. On growing the cells in the presence of PFA, the UDPGA level remains the same, suggesting that the enzyme UDPG dehydrogenase is defective, or has altered to such a form that it cannot be derepressed by growing in the presence of PFA. The former possibility is the most likely, and the suggestion is that S23 is derived from a normally mucoid strain of K12, and is non-mucoid by virtue of a defect in UDPG dehydrogenase. Many mucoid recombinants can be obtained by crossing S22, which is an Hfr strain, with S23 which is F^-, suggesting that there might be a genetic system available for the study of Colanic acid synthesis, particularly if S22M is still Hfr.

Given this genetic control of Colanic acid biosynthesis, then it is not unreasonable to suppose that at least some of the structural genes which code for enzymes in the pathway are

grouped together and form an operon, in much the same way that eight of the nine enzymes involved in the biosynthesis of the O-specific sugars in S. typhimurium map in the same region of the bacterial chromosome (Subbaiah and Stocker, 1964; Nikaido, Naide and Makela, 1966; Stocker and Wilkinson, 1966). It is unlikely that the genes coding for the synthesis of UDPG and UDPGal would be in such an operon, since these compounds are concerned in other biosynthetic reactions, including LPS synthesis. In S. typhimurium it has been shown that the genes concerned in coding for the synthesis of UDPG and UDPGal, map in a different region from those concerned with the O-specific sugars, probably a reflection of the diverse functions of these compounds, and the same applies to the gene which codes for the first enzyme involved in GDPM synthesis, phosphomannose isomerase, presumably a reflection of the fermentative function of this enzyme (Stocker and Wilkinson, 1966). However, the other enzymes in the GDPM-GDPF pathway, and UDPG dehydrogenase may well be coded for by genes which form an operon, under the control of a regulator gene, and this theory would fit well with the observed apparently simultaneous alterations in the levels of UDPGA and GDPF.

In those Salmonella strains which have mannose in the LPS in addition to fucose in Colanic acid, the question arises as to whether or not there is such a separate operon for the coding of enzymes involved in Colanic acid synthesis, including the enzymes for the synthesis of GDPM, quite separate from such

a set of genes which might code for the synthesis of GDPM as a precursor for one of the O-specific sugars. Two strains of S. typhimurium have been examined, SL1542 and SL1543. SL1542 is an Ra mutant which has only glucose, galactose, and N-acetyl glucosamine in the LPS in addition to the heptose-phosphate-KDO backbone, and it is said to have a large deletion in the his region of the chromosome, which adjoins the region which codes for the O-specific sugars, mannose, rhamnose, and abequose. The deletion is thought to include the genes which code for phosphomannomutase and GDPM pyrophosphorylase (B.A.D. Stocker, personal communication) and thus the strain has lost the ability to synthesise GDPM, and therefore GDPF. A derivative of this strain, SL1543, was obtained which appeared to be mucoid, and examination of the exopolysaccharide produced indicated that it was Colanic acid, on the basis of monosaccharide composition and sensitivity to phage depolymerases (I.W. Sutherland, unpublished results). This has been confirmed and precisely how the strain synthesises fucose has been considered. The possibility occurred that the strain may have reverted, which is unlikely (B.A.D. Stocker, personal communication), in view of the size of the deletion, and examination of the LPS of the strain indicated that it was still an Ra mutant. The fucose in Colanic acid may have been synthesised by a route other than GDPM, or it may have been synthesised via another nucleotide derivative of mannose, or the most likely explanation is that another set of enzymes exists for the synthesis of GDPF in a

Colanic acid operon. The nucleotide pools of SL1542 and SL1543 have been examined to try and detect a nucleotide derivative of fucose, but without success. SL1543 produces very little Colanic acid on ordinary media and appears to produce no more on PFA. Examination of the nucleotide pools of both show that, despite the production of a small amount of Colanic acid, only a small amount of UDPGA was detected in the soluble pool of SL1543, and very much the same amount was detected in SL1542, no fucose derivative being detected in either despite a large amount of starting material. Presumably SL1543 is only slightly de-repressed, and as such would only be expected to produce very low levels of the nucleotide sugars. Examination indicated that UDPG dehydrogenase was present at low level, and the enzymes GDPM pyrophosphorylase and GDPF synthetase were not detected. Clearly this problem requires to be further examined to establish unequivocally the source of the fucose and the genetics of the enzymes which code for its synthesis. An interesting aspect of the nucleotide pools of SL1542 and SL1543 is that both strains accumulate dTDPRh, and to a lesser extent a CDP sugar which is probably CDP abequose, the nucleotide derivative of the other O-specific sugars, so in this case presumably no feedback control mechanism exists for the control of these nucleotide sugars.

The inability to find a nucleotide derivative of the unknown sugar proposed to be a component of Colanic acid (McCleary, 1967) is not in itself significant, since frequently

sugar components of polysaccharides are not detected as nucleotide derivatives unless a pathological situation exists, causing a build up (Ginsburg, 1964). This is clearly illustrated in S53 where glucosamine and heptose are known to be components of LPS, yet nucleotide derivatives of these were not detected in the soluble pool. In some instances it is possible to demonstrate the enzymes responsible for the synthesis of a sugar nucleotide, without actually demonstrating the presence of the sugar nucleotide, but clearly in this instance, this is not possible since the structure proposed for the unknown sugar (Figure 24) is not definite as yet. In such a situation it is not possible to deduce how the compound might be synthesised, and as such, it is not clear if the unknown sugar would even exist as a nucleotide derivative in the same form, with the same chromatographic characteristics, since modification may occur at the polymer level. If the structure proposed by McCleary is correct, then the branch methyl group may not be introduced at the nucleotide sugar level, in fact it may not be introduced at all, but arise through intramolecular rearrangement. There are some recorded instances of methyl groups being added at the nucleotide sugar level in the synthesis of some of the more peculiar branched sugars and Candy, Blumson and Baddiley (1964) have produced evidence to suggest that the methyl group of S-adenosyl methionine is donated to form an N-methyl group in the synthesis of N-methyl-L-glucosamine on some nucleotide intermediates. However, the majority of cases

reported indicate that the addition of methyl groups usually occurs at the polysaccharide level, and Hassid's group (Kauss and Hassid, 1967a; 1967b; Kauss, Swanson and Hassid, 1967) have investigated the formation of O-methyl and carboxymethyl groups in various plant polysaccharides, the donor in all cases being S-adenosyl methionine. Similar work on the N and C methylation of nucleic acids has also implicated this compound (Borek and Srinivasan, 1967). However, methyl groups need not be added from some donor, and in several instances branch carbon atoms have been shown to arise by intramolecular rearrangement. This has been illustrated by labelling experiments in the biosynthesis of streptose (Candy, Blumson and Baddiley, 1964) and apiose (Mendicino and Picken, 1965) which are not dissimilar in structure to the postulated unknown sugar, and the rearrangement is postulated to take place on nucleotide derivatives, probably from dTDPG and UDPGA respectively via a 4 keto-6 deoxysugar intermediate (Wood, 1966). The structure of the unknown sugar is so uncertain at the moment, that any serious effort to investigate its synthesis must wait until more information is available.

While the nucleotide sugars involved in Colanic acid synthesis have in part at least been indicated, the precise nature of the final transferase reactions remains to be elucidated. Again the disadvantage of the system is that the structure of the polymer is so uncertain, and in particular it is not known if there is a repeating unit, which would be of

great importance in indicating how the polymer is likely to be synthesised. The possibility exists that a repeating unit does not exist, or is extremely large and complex, and the possibility of a template mechanism as postulated by Wilkinson (1958) may yet have to be considered. Ginsburg (1964) has considered the template mechanism an unnecessary complication, and suggested that the specificities of the final transferases themselves are sufficient to account for the specificity of polysaccharide synthesis. Such considerations were made before the discovery of lipid-linked intermediates, but nevertheless must not be discounted in certain circumstances until evidence to the contrary is obtained.

If a repeating unit does exist, encompassing the four known sugars and the unknown, then the hypothesis that such a unit is synthesised on some sort of lipid intermediate, is an attractive one. In general terms the existence of lipid-linked intermediates in exopolysaccharide synthesis would explain why there are no reports of mutants which synthesise altered exopolysaccharides. In all cases known, a single mutation resulting in the loss of the ability to synthesise one of the component sugars results in the deletion of the entire exopolysaccharide in an analogous way to the deletion of the entire O-specific polysaccharide in Ra mutants. Attempts have been made to isolate lipid-linked exopolysaccharide intermediates from whole cells, but without success. This may be because they do not exist, or because of the small quantities present

at any one time. The attempts which have been made to isolate the lipid-linked intermediates of LPS and murein biosynthesis from whole cells (Dankert et al., 1966; Dietrich, Colucci and Strominger, 1967) have included growing the cells in a relatively vast amount of radioactivity (2.5 mC ^{14}C-glucose or 10 mC $^{32}P_i$) and the incorporation into the intermediate is very small, only a few thousand cpm, which represents only a tiny incorporation of radioactivity. The workers involved also knew precisely what the intermediates were, having previously prepared them in cell-free systems, and they knew the chromatographic properties. In whole cells ^{14}C-glucose or $^{32}P_i$ becomes so diluted in the soluble pool that the incorporation in any compound which is present in small amount is certain to be low, and therefore difficult to detect unless very large quantities of radioactivity are available.

In the Colanic acid system it is unlikely that, if such intermediates exist, they will be found, unless a suitable cell-free system is available. In such a system with ^{14}C labelled sugar nucleotides, dilution of the radioactivity clearly does not occur, and the absence or presence of such intermediates could be determined unequivocally. Numerous attempts have been made to achieve such a system, without success. Precisely why this should be so may be for a variety of reasons. Such a system has been achieved using much the same techniques, with preparations from Klebsiella type 54 cells (I.W. Sutherland, unpublished results) where GDPM, UDPG, and UDPGA are incorporated

into a large molecular weight polymer, which is sensitive to the action of a specific depolymerase producing small molecular weight fragments (Sutherland, 1967). In such a system, incorporation from ^{14}C-UDPGA is followed into a non-dialysable compound, which is broken down to dialysable fragments by the depolymerase enzyme. This detection method for exopolysaccharide synthesis cannot be used in the Colanic acid system since none of the depolymerase enzymes available produce dialysable fragments, and in general one of the faults of the system is lack of an adequate detection method for exopolysaccharide synthesis. It would be simple and convenient to use a serological method as has been used in all the Pneumococcus cell-free systems. Purified Colanic acid has been reported to be antigenic by Goebel (1963), who managed to produce a precipitating antiserum. However, despite many attempts in this laboratory to get such an antiserum, no success has been achieved. Precisely why is not clear, perhaps the lack of antigenicity found is due to the widespread occurrence of Colanic acid synthesising enterobacteria in the test animals, and a state of immunologic paralysis exists. Samples of Goebel's antiserum have never proved satisfactory as a detection method for Colanic acid in gel precipitation tests, and the technique has been abandoned. It is unlikely to be simply the lack of a sufficiently sensitive detection method which has prevented a cell-free synthesis of Colanic acid being demonstrated, and more likely to be deficiencies in the cell-

free technique. It is a convenient hypothesis to suggest that, in view of the unknown sugar supposed to be a component of Colanic acid, cell-free synthesis has not been achieved because a suitable nucleotide derivative of the sugar was not included in the system. It might equally well be said that lipid-linked mono- and disaccharides were not detected in the cell-free system, on deletion of one or more of the nucleotide sugars, because the unknown sugar may be the first sugar to be added to the lipid, but it might equally well be argued that interpolating from an ill-defined unknown merely obscures the real faults of the system.

At the present time no one has yet isolated lipid-linked intermediates in exopolysaccharide synthesis, and while such intermediates may be eminently suitable as a method of transporting precursors of insoluble polymers across membranes, it is not yet clear if such a system would be necessary or desirable in the synthesis of soluble polymers such as Colanic acid.

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ABBREVIATIONS

Other abbreviations used in Tables, etc., not included in this list are listed beside the relevant data.

AMP	Adenosine-5′-monophosphate
ADP	Adenosine-5′-diphosphate
ATP	Adenosine-5′-triphosphate
ADPG	Adenosine-5′-diphosphate-D-glucose
$ADPGNH_2$	Adenosine-5′-diphosphate-D-glucosamine
$ADPNAcGNH_2$	Adenosine-5′-diphosphate N-acetyl-D-glucosamine
ADPM	Adenosine-5′-diphosphate-D-mannose
dADP	Deoxyadenosine diphosphate
CMP	Cytidine-5′-monophosphate
CDP	Cytidine-5′-diphosphate
CTP	Cytidine-5′-triphosphate
CMPKDO	Cytidine-5′-monophosphate 2 keto, 3 deoxy octonic acid
CMP NANA	Cytidine-5′-monophosphate N-acetyl neuraminic acid
CDPG	Cytidine-5′-diphosphate-D-glucose
$CDPGNH_2$	Cytidine-5′-diphosphate-D-glucosamine
CDPAb	Cytidine-5′-diphosphate abequose
dCDP	Deoxycytidine-5′-diphosphate
F-6-Ⓟ	α-D-fructose-6′-phosphate
GMP	Guanosine-5′-monophosphate
GDP	Guanosine-5′-diphosphate

GTP	Guanosine-5′-triphosphate
GDPM	Guanosine-5′-diphosphate-D-mannose
GDPMA	Guanosine-5′-diphosphate-D-mannuronic acid
GDPF	Guanosine-5′-diphosphate-L-fucose
GA	Glucuronic acid
G-1-Ⓟ	α-D-glucose-1-phosphate
G-6-Ⓟ	α-D-glucose-6-phosphate
Gal-1-Ⓟ	α-D-galactose-1-phosphate
IDP	Inosine-5′-diphosphate
IDPG	Inosine-5′-diphosphate-D-glucose
KDO	2 keto, 3 deoxy octonic acid
M-6-Ⓟ	α-D-mannose-6-phosphate
$NAcGNH_2$	N-acetyl-D-glucosamine
NAcMurA	N-acetyl muramic acid
$NAcMNH_2$	N-acetyl mannosamine
NANA	N-acetyl neuraminic acid
NAD (H)	Nicotinamide adenine dinucleotide (reduced)
NADP (H)	Nicotinamide adenine dinucleotide phosphate (reduced)
Ⓟ or P_i	Phosphate
PP	Pyrophosphate
dTMP	Thymidine-5′-monophosphate
dTDP	Thymidine-5′-diphosphate
dTTP	Thymidine-5′-triphosphate
dTDPG	Thymidine-5′-diphosphate-D-glucose
dTDPGal	Thymidine-5′-diphosphate-D-galactose

dTDPF	Thymidine-5′-diphosphate-L-fucose
dTDPRh	Thymidine-5′-diphosphate-L-rhamnose
$dTDPGNH_2$	Thymidine-5′-diphosphate-D-glucosamine
$dTDPNAcGNH_2$	Thymidine-5′-diphosphate N-acetyl-D-glucosamine
$dTDPNAcGalNH_2$	Thymidine-5′-diphosphate N-acetyl-D-galactosamine
UMP	Uridine-5′-monophosphate
UDP	Uridine-5′-diphosphate
UTP	Uridine-5′-triphosphate
UDPG	Uridine-5′-diphosphate-D-glucose
UDPGal	Uridine-5′-diphosphate-D-galactose
UDPGA	Uridine-5′-diphosphate-D-glucuronic acid
UDPGalA	Uridine-5′-diphosphate galacturonic acid
UDPM	Uridine-5′-diphosphate-D-mannose
$UDPGNH_2$	Uridine-5′-diphosphate-D-glucosamine
$UDPGalNH_2$	Uridine-5′-diphosphate-D-galactosamine
$UDPNAcGNH_2$	Uridine-5′-diphosphate N-acetyl-D-glucosamine
$UDPNAcGalNH_2$	Uridine-5′-diphosphate N-acetyl-D-galactosamine
UDPIA	Uridine-5′-diphosphate iduronic acid
UDPNAcMurA	Uridine-5′-diphosphate N-acetyl muramic acid
UDPX	Uridine-5′-diphosphate xylose
UDPA	Uridine-5′-diphosphate arabinose

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